CERTIFICATE OF EXPRE-
I hereby certify that the attached papers and/or fee are bei[illegible] service on the date shown below in an envelope addressed to Box P[illegible]

EH386303667US
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528 Rec'd PCT/PTO

Feb. 16, 2001
Date

Mary Rutkowski
Printed Name

Mary Rutkowski
Signature

FORM PTO-1390 (REV 12/29/99) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
PPD 50348/UST

U. S. APPLICATION NO. (If known, see 37 CFR 1.5)
09/763076

INTERNATIONAL APPLICATION NO.: PCT/GB99/02716

INTERNATIONAL FILING DATE: August 17, 1999

PRIORITY DATE CLAIMED: August 18, 1998

TITLE OF INVENTION: Genetic Method for the Expression Of Polyproteins in Plants

EXPRESS MAIL NO.: EH386303667US

APPLICANT(S) FOR DO/EO/US:
Willem BROEKAERT, Isabelle FRANCOIS, Miguel De BOLLE, Ian EVANS and John RAY

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND or SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1)).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371 (c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9 ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☒ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: PTO-1449, Copies of 11 refs. cited on PTO-1449, Paper Copy of Sequence Listing, Computer Disk Containing Sequence Listing, Statement to Support Filing and Submission Under 37 CFR 1.821-1.825

JC02 Rec'd PCT/PTO 16 FEB 2001

U.S. APPLICATION NO (If known, see 37 CFR 1 5) 09/763076	INTERNATIONAL APPLICATION NO PCT/GB99/02716	ATTORNEY'S DOCKET NUMBER PPD 50348/UST

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1. 492 (a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . $ 1000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . . . . $ 860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. . .$ 710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . .$ 690.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4). . . . .$ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$ 860.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	35-20 =	15	x $18.00	**$ 270.00**	
Independent claims	8-3 =	5	X $80.00	**$ 400.00**	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	**$**	
TOTAL OF ABOVE CALCULATIONS =				**$ 1,530.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				**$**	
SUBTOTAL =				**$ 1,530.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +					
TOTAL NATIONAL FEE =				**$ 1,530.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +					
TOTAL FEES ENCLOSED =				**$ 1,530.00**	
				Amount to be refunded:	$
				Charged	$

a. ☐ A check in the amount of $________ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **09-0013** in the amount of **$ 1,530.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **09-0013**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Syngenta Crop Protection, Inc.
Intellectual Property Section
2 Righter Parkway
P.O. Box 15458
Wilmington, DE 19850-5458

23698

PATENT TRADEMARK OFFICE

Liza D. Hohenschutz Feb. 16, 2001

SIGNATURE DATE

Liza D. Hohenschutz

NAME

33,712

REGISTRATION NUMBER

(302)476-2088

TELEPHONE NUMBER

CERTIFICATE OF EXPRESS MAIL (37 CFR 1.10) Label No. EH386303667US

I hereby certify that the attached papers and/or fee are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service on the date shown below in an envelope addressed to: Box Patent Application, Assistant Commissioner for Patents, Washington DC 20231.

Feb. 16, 2001 (Date) | Mary Rutkowski (Printed Name) | Mary Rutkowski (Signature)

PATENT APPLICATION
PPD 50348/UST

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION OF.: Willem Broekaert et al.

SERIAL NUMBER: Not Assigned **GROUP ART UNIT:** Not Assigned

FILED: **EXAMINER:** Not Assigned

FOR: Genetic Method for the Expression of Polypeptides in Plants

SECOND PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Prior to a first Official Action, please amend the above-identified patent application as follows:

IN THE SEQUENCE LISTING:

Delete the Sequence Listing (pages 1-64) and insert the Sequence Listing submitted herewith.

REMARKS

Applicants submit a paper copy of the Sequence Listing in compliance with 37 CFR 1.821-1.825, a computer disk containing the Sequence Listing in computer readable form and a Statement to Support Filing and Submission in Accordance with 37 CFR 1.821-1.825.

An early and favorable Office Action is requested.

Respectfully submitted,
Syngenta Crop Protection, Inc.

Liza D. Hohenschutz

Liza D. Hohenschutz
Reg. No. 33,712
Attorney for Applicants

Dated: February 16, 2001
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CERTIFICATE OF EXPRESS MAIL (37 CFR 1.10) Label No. EH386303667US

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Feb. 16, 2001	Mary Rutkowski	Mary Rutkowski
(Date)	(Printed Name)	(Signature)

PATENT APPLICATION
PPD 50348/UST

IN THE UNITED STATES ELECTED OFFICE

INTERNATIONAL APPLICATION NO.: PCT/GB99/02716

INTERNATIONAL FILING DATE: August 17, 1999

PRIORITY DATE CLAIMED: August 18, 1998

APPLICANTS: Willem BROEKAERT *et al.*

TITLE: Genetic Method for the Expression of Polyproteins in Plants

PRELIMINARY AMENDMENT

Box PCT
Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Prior to assigning a serial number and calculating the filing fee, please amend the above-identified application as follows:

IN THE CLAIMS

Claim 4, line 1, delete "any of the preceding claims" and substitute therefor --claim 1--.

Claim 5, line 1, delete "any of the preceding claims" and substitute therefor --claim 1--.

Claim 6, line 1, delete "any of the preceding claims" and substitute therefor --claim 1--.

Claim 7, line 1, delete "any of the preceding claims" and substitute therefor --claim 1--.

Claim 8, line 1, delete "or claim 7".

Claim 9, line 1, delete "any one of claim 7 or claim 8" and substitute therefor

--claim 7--.

Claim 14, line 1, delete "any one of the preceding claims" and substitute therefor --claim 8--.

Claim 15, line 1, delete "any one of claim 13" and substitute therefor --claim 14--.

Claim 17, line 1, delete "or 16".

Claim 18, line 1, delete "any of the preceding claims" and substitute therefor --claim 1--.

Claim 20, line 1, delete "or 3".

Claim 21, line 1, delete "any of the preceding claims" and substitute therefor --claim 1--.

Claim 24, line 1, delete "or claim 23".

Claim 25, line 3, delete "or claim 5".

Claim 29, line 1, delete "or claim 28".

Claim 30, line 1, delete "any of claims 19 to 21" and substitute therefor --claim 19--.

Claim 31, lines 1 and 2, delete "or a vector according to any one of claims 27 to 30" and substitute therefor --according to claim 27--.

REMARKS

The claims have been amended to remove improper multiple dependencies and place them in a better form for examination.

An early and favorable Office Action is requested.

Respectfully submitted,
Syngenta Crop Protection, Inc.

Liza D. Hohenschutz

Liza D. Hohenschutz
Reg. No. 33,712
Attorney for Applicants

Dated: February 16, 2001

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GENETIC METHOD FOR THE EXPRESSION OF POLYPROTEINS IN PLANTS

The present invention relates to a method for increasing protein expression levels, in particular by the coexpression of two or more proteins in plants within a single transcription unit, to the coexpression and secretion of two or more proteins in plants, to linker sequences for use in the method of the invention, to DNA constructs for use in the invention and to plants transformed with the constructs of the invention.

For many applications based on genetic modification of plants by transgenesis, it is desirable to express co-ordinately two or more transgenes. For instance, coexpression in plants of transgenes encoding antimicrobial proteins with different biochemical targets can result in enhanced disease resistance levels, resistance against a broader range of pathogens, or resistance that is more difficult to overcome by mutational adaptation of pathogens. Other examples include those aimed at producing a particular metabolite in transgenic plants by coexpression of multiple transgenes that are involved in a biosynthetic pathway. There are different ways to obtain transgenic plants expressing multiple transgenes. One frequently chosen option is to introduce each transgene individually via separate transformation events and to cross the different single-transgene expressing lines. The drawback of this method is that the different transgenes in the resulting progeny will be inserted at different loci, which complicates the subsequent breeding process. Moreover, this method is not applicable to crops that are propagated vegetatively, such as for instance potato, many ornamentals and fruit tree species.

A second possibility is to introduce the different transgenes as linked expression cassettes, each with their own promoters and terminators, within a single transformation vector. Such a set of transgenes will in this case segregate as a single genetic locus. It has been observed, however, that the presence of multiple copies of the same promoter within a transgenic plant often results in transcriptional silencing of the transgenes (Matzke, M.A. and Matzke, A.J.M., 1998, Cellular and Molecular Life Sciences 54, 94-103). In an attempt to introduce a vector containing four linked transgenes each driven by a CaMV35S promoter, Van den Elzen P.J. *et al.* (Phil. Trans. R. Soc. Lon. B., 1993, 342: 271-278) observed that none of the analysed transgenic lines expressed all four transgenes at a reasonably high level. To avoid this problem one could use different promoters for each of the expression cassettes used

in the construct. However, there is currently only a very limited choice of promoter sets that have comparable characteristics in terms of expression levels, cell-type and developmental specificity and response to environmental factors.

A third option would be to produce multiple proteins from one transcription unit by separating the distinct coding regions by so-called internal ribosomal entry sites, which allow ribosomes to reiterate translation at internal positions within a mRNA species. Although internal ribosomal entry sites are well documented in animal systems (Kaminski A. *et al.*, 1994, Genet. Eng. 16, 115-155) it is not known at present whether such sites are also functional in nuclear-encoded genes from plants. Polycistronic genes can be expressed when inserted in plant chloroplastic genomes (Daniell H. *et al.*, 1998, Nature Biotechnology 16, 345-348) but the gene products in this case are confined to the chloroplast, which is not always the preferred site of deposition of foreign proteins.

A fourth strategy, finally, is based on the production of multiple proteins by proteolytic cleavage of a single polyprotein precursor encoded by a single transcription unit. Potyviruses, for instance, translate their genomic RNA into a single polyprotein precursor that encompasses proteolytic domains able to cleave the polyprotein precursor *in cis* (Dougherty, W.G. and Carrington, J.C., 1988, Annu. Rev. Phytopathol. 26, 123-143). Beck von Bodman, S. *et al.*, (1995, Bio/Technology 13, 587-591) have already exploited the potyviral system to co-express two enzymes involved in the biosynthesis of mannopine. The two biosynthetic enzymes were fused within one open reading frame together with a protease derived from a potyviral polyprotein precursor, and the adjoining regions were separated by 8 amino acids long spacers representing specific cleavage sites for the protease. Plants transformed with this construct synthesized mannopine, suggesting that the two enzymes had somehow been produced in a form that was at least partially functional, although direct evidence for the presumed cleavage events *in planta* was not presented. A disadvantage of this system is that a viral protein needs to be co-expressed with proteins of interest, which is not always desirable. More recently, Urwin P.E. *et al.* (1998, Planta 204, 472-479) have shown that it is possible to co-express two different proteinase inhibitors joined by a protease-sensitive propeptide derived from a plant metallothionein-like protein. A polyprotein precursor consisting of a cysteine protease inhibitor (oryzacystatin from vice), a propeptide from pea metallothionein-like protein and a serine protease inhibitor (cowpea trypsin inhibitor), was found to be cleaved in transgenic

Arabidopsis thaliana plants. The cleavage, however, was only partial, as uncleaved polyprotein precursor could also be detected in the transgenic plants. As the polyprotein precursor did not contain a leader peptide, the translation products are predicted to be deposited in the cytosol. The metallothionein from which the propeptide was derived also does not contain a leader peptide (Evans IM 1990, FEBS Lett. 262, 29-32) and hence its processing must occur in the cytosol.

For some applications, cytosolic processing and deposition is a drawback. Many proteins, especially glycosylated proteins or proteins with multiple disulfide bridges, must be synthesized in the secretory pathway (encompassing the endoplasmic reticulum and Golgi apparatus) in order to be folded in a functional form (Bednarek and Raikhel 1992, Plant Mol. Biol. 20, 133-150). In addition, for some applications such as for instance the expression of antimicrobial proteins, the extracellular space is the preferred deposition site, as most microorganisms occur at least during the early stages of infection in the extracellular space. Proteins destined to the extracellular space are also synthesised via the secretory pathway but lack additional targeting information other than the leader peptide (Bednarek and Raikhel 1992, Plant Mol. Biol. 20, 133-150). Other examples of the application of this strategy are described in WO 95/24486 and WO95/17514.

The applicants have unexpectedly found that expression levels of plant defensins in plants transformed with a polyprotein precursor construct were much higher compared to those in plants transformed with single plant defensin constructs.

The invention therefore provides a method of improving expression levels of a protein in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

The processing system described here can be used not only to co-express two or more different proteins, but also to obtain higher expression levels of a protein, particularly of small proteins. The reason for the observed stimulatory effect on translational efficiency is currently

unclear. It might be due to an effect of mRNA length or length of primary translation product on translational efficiency.

Preferably, a signal sequence is operatively interconnected with the protein coding regions.

As used herein the expression "signal sequence" is used to define a sequence encoding a leader peptide that allows a nascent polypeptide to enter the endoplasmic reticulum and is removed after this translocation.

The signal sequence may be derived from any suitable source and may for example be naturally associated with the promoter to which it is operably linked. We have found the use of signal sequences from the class of plant proteins known as defensins (Broekaert et al, 1995 Plant Physiol 108, 1353-1358; Broekaert et al, 1997, Crit, Rev, Plant Sci. 16, 297-323) to be particularly suitable for use in the method of the invention.

Thus, in a further preferred embodiment, there is provided a method of improving expression levels of a protein in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

This method of the invention is particularly suitable for the expression of proteins which are 100 amino acids or less in length

The present invention provides a convenient and highly efficient method of co-expressing two or more proteins in a plant as a single transcription unit where the two proteins are joined by a cleavable linker, the construct being designed such that cleavage occurs in the secretory pathway of the plant thereby releasing the proteins extracellularly.

According to a further aspect of the present invention, there is provided a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each

other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

The two or more protein encoding regions according to all aspects of the invention preferably do not encode identical proteins i.e. the method of the invention allows the production of different proteins in a single transcription unit. The DNA sequence to be expressed according to the method of the invention is one which does not occur naturally in the plant used for the production of the multiple proteins i.e. one or more of the components of the DNA sequence will be heterologous to the plant host.

The method for the expression of multiple proteins described herein does not cover the use of a linker propeptide as expressed by the Ib-AMP gene and as described in SEQ ID Nos 14,15, 16, 17 or 18 of Published International Patent Application No. WO 95/24486 separating three protein encoding regions each of which encodes Rs-AFP2; nor the insertion thereof into a plant genome. Suitably, the method of the invention does not use a linker propetide of the native Ib-AMP gene as shown in SEQ ID Nos 14, 15, 16, 17 or 18 of WO 95/24486.

In a further aspect, the present invention there is provided a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules with the proviso that when the linker propeptide is derived from the Ib-AMP gene as described in SEQ ID Nos 14,15, 16, 17 or 18 of Published International Patent Application No. WO 95/24486 it does not separate three protein encoding regions each of which encodes Rs-AFP2.

The sequence of Rs-AFP2 is fully described in Published International patent Application no. WO 93/05153 published 18 March 1993.

The promoter sequence may for example be that naturally associated with the signal sequence, and/or it may be that naturally associated with the protein encoding sequence to which it is linked, or it may be any other promoter sequence conferring transcription in plants. It may be a constitutive promoter or it may be an inducible promoter.

The linker propeptide for use in all aspects and embodiments of the invention described herein is preferably a linker propeptide which is cleaved on passage of said DNA encoding the polyprotein precursor through the secretory pathway of the plant cells in which the polyprotein -encoding DNA is expressed. The linker propeptide is preferably designed or chosen such that cleavage of the propeptide occurs by proteases which are naturally present in the secretory pathway of the plant cell in which the DNA encoding the polyprotein is expressed. Particular promoters of the cauliflower mosaic virus such as the Penh 25S promoter of the 35S RNA, examples of such proteases include subtilisin-like proteases, .

In a preferred embodiment the invention therefore provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence, said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules said linker propeptide being cleaved on passage of said DNA encoding the polyprotein precursor through the secretory pathway of the plant cells in which the polyprotein -encoding DNA is expressed.

The method for the expression of multiple proteins described herein does not cover the use of a linker propeptide derived from the Ib-AMP gene as described in SEQ ID Nos 14, 15, 16, 17 or 18 of Published International Patent Application No. WO 95/24486 separating three protein encoding regions each of which encodes Rs-AFP2 and the insertion thereof into a plant genome.

In some embodiments of the invention, the linker propeptide is not derived from a virus.

In a particularly preferred embodiment the invention provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site

whereby the expressed polyprotein is post-translationally processed into the component protein molecules. said linker propeptide being cleaved on passage of said DNA encoding the polyprotein precursor through the secretory pathway of the plant cells in which the polyprotein -encoding DNA is expressed wherein cleavage of the propeptide occurs by proteases which are naturally present in the secretory pathway of said plant cell.

The linker propeptide may be a peptide which naturally contains processing sites for proteases occuring in the secretory pathway of plants such as the internal propeptides derived from the Ib-AMP gene which are described further herein, or may be a peptide to which such a protease processing site has been engineered at either or both ends thereof to facilitate cleavage of the sequence. Where a propeptide possesses one such protease processing site a further protease processing site may be added. If necessary or desired, repeats of the processing site, for example up to 6 repeats may be included.

For example, as described fully herein, a further protease processing site has been added to the 3' end of the DNA sequence coding for the C-terminal propeptides from Dahlia and Amaranthus which naturally possess a protease processing site at their N-terminal end for an unknown secretory pathway protease and these peptides are particularly suitable for use according to the method of the invention. Certain Dahlia sequences including C-terminal propeptide sequences are described and claimed in copending British Patent Application No. 9818003.7.

Yet another strategy is based upon the use of virus e.g. picornovirus sequences such as 20 amino acid sequences called the 2A sequence of the foot-and-mouth disease virus (FMDV) RNA, which results in the cleavage of polyproteins (Ryan and Drew 1994, EMPO J., 13, 928-933). In this instance however, in order to avoid the retention of unwanted amino acids on the protein product, combined with a sequence which produces N-terminal sequence, for example a plant derived sequence or a fragment thereof. to form a chimeric propeptide.

In the present invention. we have developed novel strategies for making artificial polyprotein precursors which are cleaved in the secretory pathway. The first one was based on the use of a propeptide derived from the IbAMP gene. IbAMP is a gene from the plant *Impatiens balsamina* which encodes a peculiar polyprotein precursor featuring a leader peptide and six consecutive antimicrobial peptides. each flanked by propeptides ranging from 16 to 28

amino acids in length (Tailor R.H. *et al.*, 1997, J. Biol. Chem. 272, 24480-24487). It is not known how and where processing of the IbAMP precursor occurs in its plant of origin. One of the internal propeptides from IbAMP was used to separate two distinct plant defensin coding regions. one originating from radish seed (RsAFP2, Terras F.R.G. *et al.*, 1992, J. Biol. Chem. 267, 15301-15309; Terras et al 1995 Plant Cell, 7, 573-588) and one from dahlia seed (DmAMP1, Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262).

An other strategy was based on the use of C-terminal propeptides from either the DmAMP1 precursor or the AcAMP2 precursor (De Bolle M.F.C. *et al.*, 1993, Plant Mol. Biol. 22, 1187-1190) or fragments of these. These C-terminal propeptides were chosen based on our previous observation that they apparently can be cleaved in transgenic tobacco plants without influencing extracellular deposition of the mature proteins to which they are connected in the precursor (R.W. Osborn and S. Attenborough, personal communication; De Bolle M.F.C. *et al.*, 1996, Plant Mol. Biol. 31, 993-1008) implicating that such cleavage is performed by a protease present in the secretory pathway excluding the vacuole. To convert these C-terminal propeptides to internal propeptides, a subtilisin-like protease processing site was engineered at the C-terminal part of the propeptides.

Subtilisin-like proteases are enzymes that specifically cleave at recognition sites of which the last two residues are basic (Barr, P.J., 1991, Cell 66, 1-3; Park C.M. *et al.*, 1994, Mol. Microbiol. 11, 155-164). Although subtilisin-like proteases are best documented in fungi (e.g. Kex2-like proteases) and higher animals (e.g. furins), recent evidence suggests that such enzymes are also present in plants (Kinal H. *et al.*, 1995, Plant Cell 7, 677-688; Tornero P. *et al.*, 1997, J. Biol. Chem. 272, 14412-14419), including Arabidopsis (Ribeiro A. *et al.*, 1995, Plant Cell 7, 785-794).

We have found that polyprotein precursors consisting of a leader peptide followed by two different plant defensins separated from each other by any of the above described internal propeptides can be processed in transgenic plants to release both plant defensins simultaneously. The cleavage does occur such that at least the major part of the plant defensins are deposited in the extracellular space. Hence processing of the precursor occurred either in the secretory pathway or in the extracellular space. The different propeptides shown to be cleaved in the transgenic plants do not reveal primary sequence homology. However, the sequences all appear to be rich in the small amino acids A, V, S and T and all contain

dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue. Although propeptide cleavage in the examples shown in this invention did apparently not occur within vacuoles, internal propeptides from vacuolar proteins (e.g. 2S albumins) might also be used if vacuolar deposition of the proteins would be desirable. In the co-expression experiments described here two different plant defensins were used but it is predicted that similar results will be obtained when other types of proteins would be used or when more than two mature protein domains would be used in the polyprotein precursor structure.

Where it is desired to target the polyprotein to a particular cellular organelle along the secretory pathway a suitable targeting sequence may be added to one or more of the multiple protein encoding regions. For example, an endoplasmic reticulum targeting sequence such as that encoding KDEL (SEQ ID NO 65) may be added to the 3' end of one or more of the mature protein encoding regions, or a vacuolar targeting sequence (Chispeels and Raikhel 1992, Cell 68, 613-616) can be added to the 3' or 5' end of one or more of the protein encoding regions. An example of the latter is the barley lectin carboxy-terminal propeptide which has been shown to destine heterologous proteins that are otherwise secreted to the vacuoles (Bednarek and Raikhel 1991, Plant Cell 3, 1195-1206; De Bolle et al, 1996 Plant Mol. Biol. 31, 993-1008).

At least 40% of the sequence of the linker propeptide for use in accordance with all aspects and methods of the invention as described herein preferably consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine.

The said hydrophobic residues are preferably alanine, valine, leucine, methionine and/or isoleucine and the said hydrophilic residues are preferably aspartic acid, glutamic acid, lysine and/or arginine.

It is further preferred that the linker propeptide has within 7 residues of its N- or C-terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

It is especially preferred that at least 40% of the sequence of the linker propeptide for use in accordance with all aspects of the invention as described herein preferably consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine and has within 7 residues of its N- or C- terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

The use of linker propeptides rich in the small amino acids A, V, S and T and containing dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue which on translation provides a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules is also preferred.

As used herein the term 'rich' is used to denote that the residues A,V, S and T are present more frequently than would be expected based on a random distribution of amino acids.

It is further preferred that the linker propeptides have a dipeptidic sequence within seven amino acids from the N- and/or C- terminal ends thereof, the said dipeptidic sequences consisting of either two acidic residues, two basic residues or an acidic and a basic residue wherein said dipeptidic sequences may be the same or different at each terminus.

In a further preferred embodiment said dipeptidic sequences are selected from the following EE, ED and/or KK.

It is particularly desirable that the linker propeptide should hold the two (or more) protein domains sufficiently far apart so that they can fold appropriately and independently. For this purpose, the linker polypeptide is suitably at least 10 and preferably at least 15 amino acids long. It is further advantageous that the linker propeptide should not interact with any secondary structural element in the two proteins which it links and should therefore itself have no particular secondary structure or form a solitary secondary structure element such as an alpha helix.

In this and all other aspects and embodiments of the invention described herein the linker propeptide sequence providing the cleavage site preferably comprises a linker sequence

which is isolatable from a natural source such as a plant or virus, or variant thereof or a frament of either of these. In particular the linker propeptide is isolatable from a plant protein, or a fragment, or variant or derivative thereof which can provide suitable cleavage sites. Particular examples include a cleavable linker derived from the C-terminal propeptide region of a Dahlia gene such as those described and claimed in copending British Patent Application No. 9818003.7.

Where a viral sequence is used, it is preferably an element of a chimeric propeptide sequence.

The expression "variant" refers to sequences of amino acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants have at least 85% similarity and preferably at least 90% similarity to the base sequence

In the context of the present invention, two amino acid sequences with at least 85% similarity to each other have at least 85% similar (identical or conservatively replaced) amino acid residues in a like position when aligned optimally allowing for up to 3 gaps, with the proviso that in respect of the gaps a total of not more than 15 amino acid residues is affected. Likewise, two amino acid sequences with at least 90% similarity to each other have at least 90% identical or conservatively replaced amino acid residues in a like position when aligned optimally allowing for up to 3 gaps with the proviso that in respect of the gaps a total of not more than 15 amino acid residues is affected.

For the purpose of the present invention, a conservative amino acid is defined as one which does not alter the activity/function of the protein when compared with the unmodified protein. In particular, conservative replacements may be made between amino acids within the following groups:

(i) Alanine, Serine, Glycine and Threonine

(ii) Glutamic acid and Aspartic acid

(iii) Arginine and Lysine

(iv) Isoleucine, Leucine, Valine and Methionine

(v) Phenylalanine, Tyrosine and Tryptophan

Sequence similarity may be calculated using sequence alignment algorithms known in the art such as, for example, the Clustal Method described by Myers and Miller (Comput. Appl. Biosci .4 11-17 (1988).) and Wilbur and Lipman (Proc. Natl. Acad. Sci. USA 80, 726-30 (1983)) and the Watterman and Eggert method (The Journal of Molecular Biology (1987) 197, 723-728). The MegAlign Lipman Pearson one pair method (using default parameters) which may be obtained from DNAstar Inc, 1228 Selfpark Street, Madison, Wisconsin, 53715, USA as part of the Lasergene system may also be used.

In particular the linker propetide is a sequence isolatable from a plant protein and more preferably from the precursor of a plant antimicrobial protein such as a defensin. or a hevein-type antimicrobial peptide (Broekaert et al 1997, Crit. Rev. Plant Sci. 16, 297-323). The linker propeptide is most preferably derivable from a defensin and/or a hevein type antimicrobial peptide, especially from the C-terminal propeptides from Dm-AMP1 and Ac-AMP2 the sequences of which are as described in Figure 2 herein (SEQ ID NO 5 and SEQ ID NO 8).

The use of a linker propeptide derived from an antimicrobial peptide derived from the genus Impatiens is also preferred. The Ib-AMP gene comprises five propeptide regions all of which are suitable for use in the present invention and which are described fully in Published International Patent Application WO 95/24486 at pages 29 and 40 to 42, the contents of which are incorporated herein by reference. All or part of the C-terminal propeptides derived from the Dm-AMP and Ac-AMP gene may be used.

In a particularly preferred embodiment, the linker propeptide sequence used comprises a naturally occurring linker propeptide sequence which is modified so that amino acids from said sequence remaining attached to protein product after cleavage thereof is reduced, preferably so that none remain. Suitable modifications may be determined using routine methods as described hereinafter. In its simplest form, protein products of the invention are isolated and analyzed to see whether they include any residual amino acids derived from the propeptide linker. The linker sequence may then be modified to eliminate some or all of these residues, provided the function of post-translational cleavage remains.

The term "fragment" refers to sequences from which amino acids have been deleted, preferably from an end region thereof. Thus these include the modified forms of the natural sequences mentioned above.

A linker propeptide of the invention may comprise one or more such fragments from different sources provided it functions as a post-translational cleavage site. Examples of linker propeptide sequences are SEQ ID NOs 3, 4, 6, 7, 21, 22, 23, 24, 25, 26, 27, 28 and 29 as shown herein and variants therefore which act as a propeptide. Particular examples of these are SEQ ID NOs 3, 4, 6, 7, 21, 22, 23, 24, 25, 26, 27, 28 and 29 themselves.

In particular, the propeptide sequences comprise SEQ ID NOs 3, 4, 6 or 7.

According to a preferred embodiment the present invention further provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide wherein the linker propeptide is derivable from a defensin and/or a hevein type antimicrobial peptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

The use of the C-terminal propeptides from Dm-AMP1 and Ac-AMP2 as described in Figure 2 herein as cleavable linkers i.e. to provide a cleavable linkage site, are particularly preferred. Depending on the choice of propeptide it may be necessary to engineer an additional specific protease recognition site at either or both ends to facilitate cleavage of the sequence. Suitable specific protease recognition sites include for example, recognition sites for subtilisin -like proteases recognising either a dipeptidic sequence consisting of two basic residues; tetrapeptidic sequences consisting of a hydrophobic residue, any residue, a basic residue and a basic residue or a tetrapeptidic sequence consisting of a basic residue, any residue, a basic residue and a basic residue. Subtilisin-like protease recognition sites are particularly preferred for use in the method of the invention.

According to a yet further preferred embodiment the present invention further provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a

signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules and wherein an additional specific protease recognition site has been engineered at either or both ends of said linker propeptide to facilitate cleavage of the sequence.

According to a yet further preferred embodiment the present invention further provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide wherein the linker propeptide is derivable from a defensin and/or a hevein type antimicrobial peptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules and wherein an additional specific protease recognition site has been engineered at either or both ends of said linker propeptide to facilitate cleavage of the sequence.

The invention further provides the use of propeptides isolatable from plant derived proteins as cleavable linkers in polyprotein precursors synthesised via the secretory pathway in transgenic plants. The propeptides are preferably isolatable from the precursor of a plant defensin or a hevein-type antimicrobial peptide (Broekaert et al 1997, Crit. Rev. Plant Sci. 16, 297-323). The propeptides may also preferably be isolatable from an antimicrobial peptide derived from the genus Impatiens.

In a further aspect the invention provides the use of a propeptide wherein at least 40% of the sequence of the propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine as a cleavable linker in polyprotein precursors synthesised via the secretory pathway in transgenic plants.

It is further preferred that the linker propeptide has within 7 residues of its N- or C-terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

It is especially preferred that at least 40% of the sequence of the linker propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine and has within 7 residues of its N- or C- terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

In a further aspect the invention provides the use of a peptide sequence rich in the small amino acids A, V, S and T and containing dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue as a cleavable linker sequence wherein said sequence is isolatable from a plant defensin or a hevein-type antimicrobial protein.

The methods of the invention may be used to achieve efficient expression and secretion of any desired proteins and is particularly suitable for the expression of proteins which must naturally be synthesised in the secretory pathway in order to be folded in a functional form such as, for example, glycosylated proteins and those with disulphide bridges. Additionally, it is extremely advantageous for proteins involved in the defence of a plant to attack by a pathogen to be secreted efficiently to the extracellular space since this is usually the initial site of pathogen attack and the present methods of the invention provide an effective means of delivering multiple proteins extracellularly.

The method of the invention is also particularly suitable for producing small peptides which may then be used for immunisation purposes i.e. the transgenic plant or a seed derived therefrom may be used directly as a foodstuff thereby passively immunising the recipient.

Examples of proteins which may be expressed according to the methods of the present invention include, for example, antifungal proteins described in Published International Patent Application Nos WO92/15691, WO92/21699, WO93/05153, WO93/04586, WO94/11511, WO95/04754, WO95/18229, WO95/24486, WO97/21814 and WO97/21815 including Rs-AFP1, Rs-AFP2, Dm-AMP1, Dm-AMP2, Hs-AFP1, Ah-AMP1, Ct-AMP1, Ct-AMP2, Bn-

AFP1, Bn-AFP2, Br-AFP1, Br-AFP2, Sa-AFP1, Sa-AFP2, Cb-AMP1, Cb-AMP2, Ca-AMP1, Bm-AMP1, Ace-AMP1, Ac-AMP1, Ac-AMP2, Mj-AMP1, Mj-AMP2, Ib-AMP1, Ib-AMP2, Ib-AMP3, Ib-AMP4, PR-1 type proteins such as chitinases, glucanases such as beta1,3 and beta1,6 glucanases, chitin-binding lectins, zeamatins, osmotins, thionins and ribosome-inactivating proteins and peptides derived therefrom or antifungal proteins showing 85% sequence identity, preferably greater than 90% sequence identity, more preferably greater than 95% sequence identity with any of said proteins where sequence identity is as defined above.

The cleavable linkers are used to join two or more proteins of interest and provide cleavage sites whereby the polyprotein is post-translationally processed into the component protein molecules.

In a further aspect the invention provides a DNA construct comprising a DNA sequence comprising a promoter region operably linked to a plant derived signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a post-translational cleavage site.

Suitably the protein encoding region encode different proteins. Preferred examples of propeptide linker sequences are as detailed above.

In a preferred embodiment of this aspect the invention provides a DNA construct wherein said DNA sequence encoding said linker propeptide encodes an internal propeptide from the Ib-AMP gene. In a further preferred embodiment of this aspect the invention provides a DNA construct wherein said DNA sequence encoding said linker propeptide encodes the C-terminal propeptide from the Dm-AMP or from the Ac-AMP gene.

In a particularly preferred embodiment the invention provides a DNA construct as described above wherein when the DNA sequence encoding the linker propeptide is derived from the Dm-AMP gene or from the Ac-AMP gene it additionally comprises one or more protease recognition sites at either or both ends thereof.

In a further aspect the invention provides a DNA construct comprising a DNA sequence comprising a promoter region operably linked to two or more protein encoding regions and a 3' terminator-region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide encoding the C-terminal

propeptide from the Dm-AMP gene or the from the Ac-AMP gene said propeptide providing a post-translational cleavage site.

In a particularly preferred embodiment the invention provides a DNA construct as described above wherein the DNA sequence encoding the linker propeptide from Dm-AMP or Ac-AMP additionally comprises one or more protease recognition sites at either or both ends thereof.

In a yet further aspect the invention provides a transgenic plant transformed with a DNA construct according to any of the above aspects of the invention.

In a further aspect the invention provides a transgenic plant transformed with a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide which on translation provides a cleavage site.

In a preferred embodiment of this aspect at least 40% of the sequence of the said linker propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine.

The said hydrophobic residues are preferably alanine, valine. leucine, methionine and/or isoleucine and the said hydrophilic residues are preferably aspartic acid, glutamic acid, lysine and/or arginine.

It is further preferred that the linker propeptide has within 7 residues of its N- or C-terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

It is especially preferred that at least 40% of the sequence of the linker propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine. arginine. histidine, serine, threonine, glutamine and asparagine and has within 7 residues of its N- or C- terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

In a further preferred embodiment of this aspect of the invention the DNA sequence providing the cleavage site encodes a peptide sequence rich in the small amino acids A, V, S and T and containing dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue.

In a particularly preferred embodiment of this aspect of the invention the DNA sequence providing the cleavage site encodes a propeptide derived from the Ib-AMP gene such as for example that described in Figure 2. In a further particularly preferred embodiment of this aspect of the invention the DNA sequence providing the cleavage site encodes the C-terminal propeptides from Dm-AMP1 and Ac-AMP2 as described in Figure 2 which may optionally be engineered to include a further DNA sequence encoding a subtilisin-like protease recognition site.

In a further aspect the invention provides a vector comprising a DNA construct as described above.

Certain linker sequences described herein are novel and theses and the coding sequence for these form a further aspect of the invention. In particular therefore, there is provided a nucleic acid which encodes a linker peptide of SEQ ID NO 4, 6, 7, 29, 21, 22, 23, 24, 25, 26, 27, 28 or the linker peptide shown in Figure 34 as well as variants thereof. Particular variants will be those which have SEQ ID NO 77 linked at the C-terminal end.

As will be readily apparent to a man skilled in the art the sequence of the individual components of the DNA sequence i.e. the signal sequence, promoter sequence, linker sequence, protein sequence(s), terminator sequence for use in the methods according to the invention may be predicted from its known amino acid sequence and DNA encoding the protein may be manufactured using a standard nucleic acid synthesiser. Alternatively, DNA encoding the components of the invention may be produced by appropriate isolation from natural sources.

The invention is further illustrated with reference to the following non-limiting examples and figures in which

Figure 1: shows nucleotide sequence (SEQ ID NO 1) and corresponding amino acid sequence (SEQ ID NO 2) of coding region of the DmAMP1 gene. The amino acids corresponding to mature DmAMP1 are underlined. The nucleotides corresponding to the intron are double underlined.

Figure 2: shows schematic representation of the coding regions from the vector constructs (SEQ ID NOS 3-8). Amino acids sequences below the internal propeptides represent the propeptide sequences from which the linker propeptides were derived.

Figure 3: shows schematic representation of plant transformation vector pFAJ3105

Figure 4: shows schematic representation of plant transformation vector pFAJ3106

Figure 5: shows schematic representation of plant transformation vector pFAJ3107

Figure 6: shows schematic representation of plant transformation vector pFAJ3108

Figure 7: shows schematic representation of plant transformation vector pFAJ3109

Figure 8: shows nucleotide sequence (SEQ ID NO 9) and corresponding amino acid sequence (SEQ ID NO 10) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3105. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively.

Figure 9: shows nucleotide sequence (SEQ ID NO 11) and corresponding amino acid sequence (SEQ ID NO 12) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3106. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively.

Figure 10: shows nucleotide sequence (SEQ ID NO 13) and corresponding amino acid sequence (SEQ ID NO 14) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3107. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively.

Figure 11: shows nucleotide sequence (SEQ ID NO 15) and corresponding amino acid sequence (SEQ ID NO 16) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3108. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively.

Figure 12: shows nucleotide sequence (SEQ ID NO 19) and corresponding amino acid sequence (SEQ ID NO 20) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3109. The amino acids corresponding to mature DmAMP1 are underlined.

Figure 13: shows the Dm-AMP1 expression levels (as % of total soluble protein) of a series of transgenic individual plants transformed with construct pFAJ3105 and a series of transgenic individuals transformed with construct pFAJ3109.

Figure 14: shows RP-HPLC analysis on a C8-silica column of crude extracts from leaves transformed with construct pFAJ3105 (A) or pFAJ3106 (B). Extracts were prepared as described in Materials and Methods. The column was eluted with a gradient of acetonitrile in 0.1 % TFA (0-35 min. 15 % - 50 % acetonitrile in 0.1 % TFA). The eluate was monitored on-line for measurement of the absorbance at 214 nm (top trace), fractionated, and subjected to Elisa assays for DmAMP1 (lower bar graph, black bars) and RsAFP2 (lower bar graph, white bars). The elution position of authentic DmAMP1 and RsAFP2 are indicated with arrows on the A_{214} chromatograms.

Figure 15: shows the results of reverse phase chromatography (RPC) of the extracellular fluid fraction of Arabidopsis plants transformed with construct 3105 (line 14). RPC was performed on a C8-silica column (Microsorb-MV, 4.6 x 250 mm, Rainin) equilibrated with 0.1 % trifluoroacetic acid (TFA). After loading the column was eluted at a flow rate of 1 ml/min for 20 min with 0.1 % TFA, whereafter a 35 min linear gradient was applied from 15 to 50 % acetonitrile in 0.1 % TFA. Absorbance (full line) was measured on-line at 280 nm and acetonitrile concentration (dashed line) was measured on-line with a conductivity monitor. Fractions were collected and assessed for DmAMP1-CRP and RsAFP2-CRP using ELISA assays. Peak numbers in bold indicate presence of DmAMP1-CRP, peak numbers in italic indicate presence of RsAFP2-CRP.

Figure 16: shows the results of RPC of an extract of Arabidopsis plants transformed with construct 3105 (line 14). Samples were two different fractions from IEC showing presence of either DmAMP1-CRPs or RsAFP2-CRPs, namely those fractions eluting between 0.17 – 0.33 M NaCl (A), and 0.33 – 0.49 M NaCl (B). RPC was performed as in the legend to Figure 14. Absorbance (full line) was measured on-line at 280 nm and acetonitrile concentration (dashed line) was measured on-line with a conductivity monitor. Fractions were collected and assessed for DmAMP1-CRP or RsAFP2-CRP using ELISA assays. Peak numbers in bold indicate presence of DmAMP1-CRP, peak numbers in italic indicate presence of RsAFP2-CRP.

Figure 17: shows the amino acid sequence of the polyprotein precursors encoded by constructs pFAJ3105, pFAJ3106 and pFAJ3108. Dashes indicate omission from the full sequence for sake of brevity. The sequence in italic is the DmAMP1 leader peptide. the underlined sequence is mature DmAMP1, the bold sequence is the linker peptide. the double underlined

sequence is mature RsAFP2. Arrows indicate processing sites according to the N-terminal sequence and mass spectrometry analyses of purified DmAMP-CRPs and RsAFP2-CRPs.

Figure 18: shows the RPC of the extracellular fluid fraction of Arabidopsis plants transformed with construct pFAJ3106 (line 9). RPC was performed and fractions analysed as described in the legend to figure 15. Peak numbers in bold indicate presence of DmAMP1-CRP, peak numbers in italic indicate presence of RsAFP2-CRP.

Figure 19: shows the RPC results of an extract of Arabidopsis plants transformed with construct 3108 (line 9). The sample was a fraction from IEC showing presence of either DmAMP1-CRPs or RsAFP2-CRPs, namely those fractions eluting between 0.17 – 0.33 M NaCl and showing the presence of DmAMP1-CRPs. RPC was performed and fractions analysed as in the legend to Figure 15. Peak numbers in bold indicate presence of DmAMP1-CRP.

Figure 20: is a schematic representation of the coding region of constructs pFAJ3105, pFAJ3343, pFAJ3344, pFAJ3345, pFAJ3346, and pFAJ3369. Full arrowheads indicate experimentally determined cleavage sites. Open arrowheads indicate presumed cleavage sites. Abbreviations: SP DmAMP1: signal peptide region of DmAMP1 (see figure 1); DmAMP1: mature protein region of DmAMP1 (see figure 1); RsAFP2: mature protein region of RsAFP2 (Terras et al. 1995, Plant Cell, 7, 573-588). Linker peptide sequences are shown in full (SEQ ID NOS 3, 29, 21-24 respectively).

Figure 21: is a schematic representation of the coding region of constructs pFAJ3367 with linker peptide of SEQ ID NO 24. Abbreviations: SP DmAMP1: signal peptide region of DmAMP1 (see figure 1); DmAMP1: mature protein region of DmAMP1 (see figure 1); RsAFP2: mature protein region of RsAFP2 (Terras et al. 1995, Plant Cell, 7, 573-588); HsAFP1: mature protein region of HsAFP1 (Osborn et al. 1995, FEBS Lett. 368, 257-262); AceAMP1; mature protein region of AceAMP1 (Cammue et al. 1995, Plant Physiol. 109, 445-455).

Figure 22: is a schematic representation of the coding region of constructs pFAJ3106-2, pFAJ3107-2, and pFAJ3108-2. Abbreviations: SP DmAMP1: signal peptide region of DmAMP1 (see figure 1); DmAMP1: mature protein region of DmAMP1 (see figure 1);

RsAFP2: mature protein region of RsAFP2 (Terras et al. 1995, Plant Cell, 7, 573-588); RS Kex2p: recognition sequence (IGKR) of the Kex2 protease (Jiang and Rogers, 1999, Plant J., 18, 23-32); AcAMP1: mature protein region of AcAMP1 (De Bolle et al. Plant Mol Biol, 31, 997-1008). The linker propeptide sequences are shown in full as SEQ ID NOS 25, 26 and 27 respectively.

Figure 23: is a schematic representation of the coding region of constructs pFAJ3368 and pFAJ3370. Open arrowheads indicate presumed cleavage sites. Abbreviations: SP DmAMP1: signal peptide region of DmAMP1 (see figure 1); DmAMP1: mature protein region of DmAMP1 (see figure 1); RsAFP2: mature protein region of RsAFP2 (Terras et al. 1995, Plant Cell, 7. 573-588); 2A sequence: cleavage recognition site of the Foot and Mouth Disease Virus polyprotein. The linker propeptide sequence is shown in full as SEQ ID NO 28.

Figure 24: shows nucleotide sequence (SEQ ID NO 30) and corresponding amino acid sequence (SEQ ID NO 31) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3343. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 29).

Figure 25: shows the nucleotide sequence (SEQ ID NO 32) and corresponding amino acid sequence (SEQ ID NO 33) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3344. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 21).

Figure 26: shows the nucleotide sequence (SEQ ID NO 34) and corresponding amino acid sequence (SEQ ID NO 35) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3345. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 22).

Figure 27: shows the nucleotide sequence (SEQ ID NO 36) and corresponding amino acid sequence (SEQ ID NO 38) of the open reading frame of the region comprised between the

*Nco*I and *Sac*I sites of plasmid pFAJ3346. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 23).

Figure 28: shows the nucleotide sequence (SEQ ID NO 38) and corresponding amino acid sequence (SEQ ID NO 39) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3369. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 24) .

Figure 29: shows the nucleotide sequence and corresponding amino acid sequence of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3367. The amino acids corresponding to mature DmAMP, mature RsAFP2, mature HsAFP1 and mature AceAMP1 are underlined, double-underlined, dashed-underlined and dotted-underlined, respectively. The amino acids corresponding to the internal linker peptides are in bold (SEQ ID NO 24).

Figure 30: shows the nucleotide sequence (SEQ ID NO 42) and corresponding amino acid sequence (SEQ ID NO 43) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3106-2. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 4).

Figure 31: shows the nucleotide sequence (SEQ ID NO 44) and corresponding amino acid sequence (SEQ ID NO 45) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3107-2. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 6).

Figure 32: shows the nucleotide sequence (SEQ ID NO 46) and corresponding amino acid sequence (SEQ ID NO 47) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3108-2. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 7).

Figure 33: shows the nucleotide sequence (SEQ ID NO 48) and corresponding amino acid sequence (SEQ ID NO 49) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3370. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The linker sequence is indicated in bold type (SEQ ID NO 28) with the amino acids corresponding to the 2A sequence indicated in bold italic.

Figure 34: shows the nucleotide sequence (SEQ ID 48) and corresponding amino acid sequence (SEQ ID NO 49) of the open reading frame of the region comprised between the *Nco*I and *Sac*I sites of plasmid pFAJ3368. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The linker sequence is indicated in bold type with amino acids corresponding to the 2A sequence are indicated in bold italic.

The following Examples illustrate the invention.

Example 1

Cloning of DmAMP1 cDNA and DmAMP1 gene

Cloning procedures and polymerase chain reaction (PCR) procedures were performed following standard protocols (Sambrook *et al*., 1989, Molecular Cloning: a laboratory manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). A cDNA library was constructed from near-dry seeds collected from flowers of *Dahlia merckii*. Total RNA was purified from the seeds using the method of Jepson I. *et al*. (1991, Plant Mol. Biol. Reporter 9, 131-138). 0.6 mg of total RNA was obtained from 2 g of *D. merckii* seed. PolyATract magnetic beads (Promega) were used to isolate approximately 2 µg poly-A+ RNA from 0.2 mg of total RNA.

The poly-A+ RNA was used to construct a cDNA library using a ZAP-cDNA synthesis kit (Stratagene). Following first and second strand synthesis, cDNAs were ligated with vector DNA. After phage assembly using Gigapack Gold (Stratagene) packaging extracts, approximately 1 x 10^5 plaque forming units (pfu) were obtained.

Using oligonucleotides AFP-5 (5'-TG(T,C)GANAANGCN(A,T)(G,C)NAA(A,G)ACNTGG)

(SEQ ID NO 13) based on the N-terminal sequence CEKASKTW (SEQ ID NO 14) of DmAMP1, Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262) and AFP-3EX (5'-CA(A,G)TT(A,G)AANTANCANAAA(A,G) CACAT) (SEQ ID NO 52) based on the C-terminal sequence MCFCYFNC (SEQ ID NO 53) of DmAMP1) and genomic DNA isolated from *D. merckii* leaves. a 144 bp PCR product was produced and isolated from an agarose gel. The PCR product was cloned into pBluescript. The insert of 10 transformants were sequenced. The sequences represented 3 closely homologous DmAMP1-like genes one of which, PCR clone 4, encoded the observed mature DmAMP1. The 144 bp PCR product mixture labelled with ^{32}-P CTP was used to probe Hybond N (Amersham) filter lifts made from plates containing a total of 6×10^4 pfu of the cDNA library. Thirty potentially positive signals were observed. 22 plaques were picked and taken through two further rounds of screening. After in vivo excision 13 clones were characterised by DNA sequencing. Four classes of DmAMP related peptides were encoded by the 13 cDNA clones. Three versions of the DmAMP mature protein region were represented in the four classes. One of the classes (Dm2.5 type) contained a mature protein region which may correspond to DmAMP2 (Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262). None of the cDNAs encoded a mature protein region equivalent to the observed mature DmAMP1 peptide sequence.

Using the sequence of PCR clone 4 (above) and information from the N- and C-terminal ends of the peptides deduced from cDNA sequences, two pairs of oligonucleotides were designed for amplification of a gene encoding DmAMP1. Genomic DNA from *D. merckii* was used in a PCR reaction with oligonucleotides MATAFP-5P (5'-ATGGC(C,G)AAN(A,C)(A,G)NTC (A,G)GTTGCNTT) (SEQ ID NO 66) and MATAFP-5 (5'- AAACACATGTGTTTCCCATT) (SEQ ID NO 54), the PCR product was cloned into pBluescript and clones were sequenced. A clone containing the 5' half of a DmAMP1 gene was identified. Genomic DNA from *D. merckii* was used in a PCR reaction with MATAFP-3 (5'- AGCGTGTCATGTGCGTAAT) (SEQ ID NO 55) and DM25MAT-3 (5'- TAAAGA AACCGACCCTTTCACGG) (SEQ ID NO 56), the PCR product was cloned into pBluescript and clones were sequenced. A clone containing the 3' half of a DmAMP1 gene was identified. The 5' and 3' sections of the mature gene were combined to assemble the sequence of the coding region of the DmAMP1 gene (Figure 1).

The DmAMP1 gene encodes a precursor with a 28 amino acids leader peptide, a 50 amino acids mature protein and a 40 amino acids C-terminal propeptide. The open reading frame is interrupted by a 92 bp intron located within the leader peptide region.
To eliminate the intron from the DmAMP1 gene sequence and to allow cloning of the DmAMP1 encoding region, either with or without the C-terminal propeptide region, into an expression cassette vector, two PCR reactions were carried out with respectively the primer sets DMVEC-3 (5'- ATGCATCCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTT CTGATCCTTTTCGTGCTCGCCATCTCAGATATCGCATCCGTTAGTGGAGAACTATG CGAGAAA) (SEQ ID NO 57) and DMVEC-2 (5'- AAACCGACCGAGCTCACGGATGTTCAACGTTTGGA AC) (SEQ ID NO 58), and DMVEC-3 and DMVEC4 (5'- AGCAAGCTTTTCGGGAGCTCAACAATTGA AGTAA)(SEQ ID NO 59). DMVEC-3 primes at the top strand of the DmAMP1 gene, corresponds to the leader peptide region without the intron and introduces an *Nco*I site at the translation start. DMVEC-2 primes at the bottom strand of the DmAMP1 gene at the 3'-end of the C-terminal propeptide region and introduces a *Sac*I site behind the translation stop codon. DMVEC-4 primes at the bottom strand of the DMAMP1 gene at the 3' end of the mature protein region, fuses a stop codon behind this region and introduces a *Sac*I site behind the stop codon.

Both PCR products were cut with *Nco*I and *Sac*I which cleaved the PCR products in two fragments due to an internal *Nco*I site in the mature protein region. The resulting *Nco*I-*Sac*I and *Nco*I-*Nco*I fragments were cloned sequentially in plasmid pMJB1. pMJB1 is an expression cassette vector containing in sequence a *Hin*dIII site, the enhanced cauliflower mosaic 35S RNA (CaMV35S) promoter (Kay R. *et al.*, 1987, Science 236, 1299-1302), a *Xho*I site, the 5' untranslated leader sequence of tobacco mosaic virus (TMV) (Gallie D.R. and Walbot V., 1992, Nucl. Ac. Res. 20, 4631-4638) a polylinker including *Nco*I, *Sma*I, *Kpn*I and *Sac*I sites, the 3' untranslated terminator region of the *Agrobacterium tumefaciens* nopaline synthase gene (Bevan M.W. *et al.*, 1983, Nature 304, 184-187) and an *Eco*RI site. The resulting plasmids were termed pDMAMPE (leader peptide region, mature protein region and C-terminal propeptide region) and pDMAMPD (leader peptide region and mature protein region), respectively. The coding regions were verified by DNA sequencing.

Example 2

Construction of plant transformation vectors

To explore the possibility of expressing polyprotein precursor genes in plants, four different plant transformation vectors were made with the aim to co-express two different cysteine-rich plant defensins with antifungal properties, namely RsAFP2 and DmAMP1. The polyprotein precursor regions of these constructs all featured a leader peptide region derived from the DmAMP1 cDNA, the mature protein domain of DmAMP1, an internal propeptide region, and the mature protein domain of RsAFP2. The four constructs differed only in the internal propeptides (Figure 2):

- construct 3105 has one of the IbAMP internal propeptides as a propeptide separating DmAMP1 and RsAFP2.
- construct 3106 has a propeptide consisting of a part of the DmAMP1 propeptide and a putative subtilisin-like protease processing site (IGKR) (SEQ ID NO 67) at its C-terminus.
- construct 3107 is identical to construct 3106 except that the entire DmAMP1 propeptide was taken.
- construct 3108 has a propeptide consisting of the AcAMP2 propeptide and a putative subtilisin-like protease processing site (IGKR) at its C-terminus.

The rationale behind constructs 3106, 3107 and 3108 is based on our observations that the C-terminal propeptides of AcAMP2 and DmAMP1 are cleaved off at their N-terminus when expressed as AcAMP2- and DmAMP1-preproproteins in tobacco, respectively, while this processing event does not detract the mature proteins from being sorted to the apoplast (De Bolle *et al*., 1996, Plant Mol. Biol. 31, 993-1008; R.W. Osborn and S. Attenborough, personal communication). This infers that the processing enzymes are either in the secretory pathway or in the apoplast. On the other hand, C-terminal cleavage of the internal propeptide in these constructs should be executed by a subtilisin-like protease, a member of which in yeast (Kex2) is known to occur in the Golgi apparatus (Wilcox C.A. and Fuller R.S., 1991, J. Cell. Biol. 115, 297), while a member in tomato occurs in the apoplast (Tornero P. *et al*., 1997, J. Biol. Chem. 272, 14412-14419). Proteins deposited in the apoplast, the preferred deposition site for antimicrobial proteins engineered in transgenic plants (Jongedijk E. *et al*., 1995, Euphytica 85, 173-180; De Bolle *et al*., 1996, Plant Mol. Biol. 31, 993-1008) are normally synthesized via the secretory pathway, encompassing the Golgi apparatus.

A construct was also made for expression of only DmAMP1 (construct 3109, figure 7).

Schematic representations of the plant transformation vectors prepared, pFAJ3105, pFAJ3106, pFAJ3107, pFAJ3108 and pFAJ3109, are shown in Figures 3 to 7, respectively. The nucleotide sequences comprised between the *Xho*I and *Sac*I sites of these plasmids, which encompass the regions encoding antimicrobial proteins, are presented in Figures 8 to 13. The regions comprised between the *Xho*I and *Sac*I sites of plasmid pFAJ3105 (shown in Figure 8) was constructed following the two-step recombinant PCR protocol of Pont-Kindom G.A.D. (1994, Biotechniques 16, 1010-1011). Primers OWB175 (5'AGGAAGTTCATTTCATTTGG) and (SEQ ID NO 68), OWB278 (5'-GCCTTTGGCACAACTTCTGT CCTGGCTCCACGTCCTCTGGGGTAGCCACCTCGTCAGCAGCGTTGGAACAATTGA AGTAACAGAAACAC) (SEQ ID NO 60) were used in a first PCR reaction with plasmid pDMAMPE (see above) as a template. The second PCR reaction was done using as a template plasmid pFRG4 (Terras F.R.G. *et al.*, 1995, Plant Cell 7, 573-588) and as primers a mixture of the PCR product of the first PCR reaction, primer OWB175 and primer OWB172 (5'TTAGAGCTCCTATTAACAAGGAAAGTAGC (SEQ ID NO 61), *Sac*I site underlined). The resulting PCR product was digested with *Xho*I and *Sac*I and cloned into the expression cassette vector pMJB1 (see above). The expression cassette in the resulting plasmid, called pFAJ3099, was digested with *Hin*dIII (flanking the 5' end of the CaMV35S promoter) and *Eco*RI (flanking the 3' end of the nopaline synthase terminator) and cloned in the corresponding sites of the plant transformation vector pGPTVbar (Becker D. *et al.*, 1992, Plant Mol. Biol. 20, 1195-1197) to yield plasmid pFAJ3105.

Plasmids pFAJ3106, pFAJ3107 and pFAJ3108 were constructed analogously except that primer OWB278 in the first PCR reaction was replaced by the following primers, respectively: OWB279 (5'-GCCTTTGGCACAACTTCTGCCTCTTTCCGATGAGTTGTTCGGCTTT AAGTTTGTC); (SEQ ID NO 62), OWB303 (5'-GCCTTTGGCACAACTTCTGCCTCTTTCCG ATCGGATGTTCAACGTTTGGAACC) (SEQ ID NO 63) : OWB304 (5'-GCCTTTGGCACAACTTCTGCCT CTTTCCGATAGTTTTGGTGGCAGCAACATCAGCTTGGTGATCCACAGTAGTACTGG CACAATTGAAGTAACAGAAACAC) (SEQ ID NO 64).

Plasmid pFAJ3109 was constructed by cloning the *Hin*dIII-*Eco*RI fragment of plasmid pDMAMPD (see above) into the corresponding sites of plant transformation vector pGPTVbar (see above).

Example 3

Plant transformation

Arabidopsis thaliana ecotype Columbia-O was transformed using recombinant *Agrobacterium tumefaciens* by the inflorescence infiltration method of Bechtold N. *et al.* (1993, C.R. Acad. Sci. 316, 1194-1199). Transformants were selected on a sand/perlite mixture subirrigated with water containing the herbicide Basta (Agrevo) at a final concentration of 5 mg/l for the active ingredient phosphinothricin.

Example 4

Assays for target proteins including Elisa assays and protein assays

Antisera were raised in rabbits injected with either RsAFP2 (purified as described in Terras F.R.G. *et al.*, 1992, J. Biol. Chem. 267, 15301-15309) or DmAMP1 (purified as in Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262). ELISA assays were set up as competitive type assays essentially as described by Penninckx I.A.M.A. *et al.* (1996, Plant Cell 8, 2309-2323). Coating of the ELISA microtiter plates was done with 50 ng/ml RsAFP2 or DmAMP1 in coating buffer. Primary antisera were used as 1000- and 2000-fold diluted solutions (DmAMP1 and RsAFP2, respectively) in 3 % (w/v) gelatin in PBS containing 0.05 % (v/v) Tween 20.

Total protein content was determined according to Bradford (1976, Anal. Biochem. 72, 248-254) using bovine serum albumin as a standard.

Arabidopsis leaves were homogenized under liquid nitrogen and extracted with a buffer consisting of 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 1.5 M NaCl. The homogenate was heated for 10 min at 85°C and cooled down on ice. The heat-treated extract was centrifuged for 15 min at 15 000 x *g* and was injected on a reserved phase high pressure liquid chromatography column (RP-HPLC) consisting of C8 silica (0,46 cm x 25 cm; Rainin) equilibrated with 0.1 % (v/v) trifluoroacetic acid (TFA). The column was eluted at 1 ml/min in a linear gradient in 35 min from 15 % to 50 % (v/v) acetonitrile in 0.1 % (v/v) TFA. The

eluate was monitored for absorbance at 214 nm, collected as 1 ml fractions, evaporated and finally redissolved in water. The fractions were tested by ELISA assays.

Example 5

Preparation of intracellular extract

Intercellular fluid was collected from Arabidopsis leaves by immersing the leaves in a beaker containing extraction buffer (10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 1.5 M NaCl). The beaker with the leaves was placed in a vacuum chamber and subjected to six consecutive rounds of vacuum for 2 min followed by abrupt release of vacuum. The infiltrated leaves were gently placed in a centrifuge tube on a grid separated from the tube bottom. The intercellular fluid was collected from the bottom after centrifugation of the tubes for 15 min at 1800 x *g*. The leaves were resubjected to a second round of vacuum infiltration and centrifugation and the resulting (extracellular) fluid was combined with that obtained after the first vacuum infiltration. After this step the leaves were extracted in a Phastprep (BIO101/Savant) reciprocal shaker and the extract clarified by centrifugation (10 min at 10,000 x *g*) and the resulting supernatant considered as the intracellular extract.

Expression levels of DmAMP1 and RsAFP2 were analysed in leaves taken from a series of T1 transgenic Arabidopsis plants resulting from transformation with the constructs described above. The results of the expression analyses based on Elisa assays as described above are presented in Table 1.

Table 1: Expression levels of Dm-AMP1 and Rs-AFP2 in transgenic Arabidopsis lines

construct	line	Expression level of Dm-AMP1 (%)	expression level of Rs-AFP2 (%)	construct	line	Expression level of Dm-AMP1 (%)	expression level of Rs-AFP2 (%)
3105	1	0,77	0,29	**3107**	1	0,04	0.04
	2	1,13	0.22		2	0,75	0,42
	3	0.48	0.20		3	0,14	0.13
	4	0,005	<0.001		4	0,01	0,01
	5	0,36	0,05		5	0,27	0,29
	6	0,99	0,25	**3108**	1	0,47	0,10
	7	0,60	0,09		2	3,00	0,53
	8	0,13	<0,001		3	0,91	0,24
	9	0,25	0,08		4	2,04	0.22
	10	4,15	0,85		5	0,17	0,04
	11	1,35	0,35		6	0,55	0,05
	12	0,24	0,07		7	0,16	0,11
	13	4,43	0,91		8	0,05	0,02
	14	1,18	0,24		9	0,45	0,02
	15	0,68	0,17	**3109**	1	0,19	nd
	16	0,49	0,07		2	0,05	nd
3106	1	0,10	0,001		3	0,02	nd
	2	1,82	0,008		4	0,20	nd
	3	0,68	0,20		5	0,10	nd
	4	1,15	0,38		6	0,06	nd
	5	0,20	0,10		7	0,07	nd
	6	0,10	0,05		8	0.003	nd
	7	0,40	0,17		9	0,18	nd
	8	2,64	0,50				
	9	0,40	0,15				
	10	0,21	0,07				
	11	0,06	0,03				
	12	0,24	0,09				

In the above Table "nd" indicates not done.

Most of the tested lines transformed with the polyprotein constructs 3105, 3106, 3107 and 3108 clearly expressed both DmAMP1-CRPs (DmAMP1-crossreactive proteins) and RsAFP2-CRPs (Rs-AFP2-crossreactive proteins). There was generally a good correlation between DmAMP1-CRP and RsAFP2-CRP levels. However, the RsAFP2-CRP levels were generally 2 to 5-fold lower than the DmAMP1-CRP levels. The Elisa assays for measuring the RsAFP2-CRPs in the extracts are, however, less reliable than those for the Dm-AMP1-CRPs. In Rs-AFP2 Elisa assays, dilutions of extracts of transgenic plants yielded dose-response curves that deviated from those obtained for dilutions of standard solutions containing authentic Rs-AFP2, indicating that the majority of the Rs-AFP2 -CRPs in the extracts were imunologically not identical to RsAFP2 itself. Deviations from RsAFP2 standard dose-response curves were much more pronounced for extracts from plants transformed with constructs 3106, 3107, and 3108 than for those of plants transformed with 3105.

None of the extracts showed deviations from Dm-AMP1 standards in dose response curves in Dm-AMP1 Elisa assays. The DmAMP-CRP levels in the lines transformed with the polyprotein constructs 3105, 3106, 3107 or 3108 were generally much higher compared to those in the line transformed with the single protein construct 3109. This is also illustrated in Figure 13 where DmAMP1-CRP expression levels are compared for plants transformed with the polyprotein construct 3105 and plants transformed with the single protein construct 3109. Expression levels as high as 4% of total protein (e.g. DmAMP1-CRP level in lines 3105-15 and 3105 -18, see table 1) have so far never been reported in the literature for a peptide expressed in transgenic plants. Hence, the use of polyprotein constructs appears to result in markedly enhanced expression, which is an unexpected finding.

Example 6

Separation of proteins processed from polyprotein precursors

A transgenic line was selected among each of the populations transformed with either construct 3105 (line 1) or 3106 (line 2) and the selected lines were further bred to obtain plants homozygous for the transgenes. In order to analyse whether DmAMP1 and RsAFP2 were correctly processed in these lines, extracts from the plants were prepared as described in Example 1 and separated by RP-HPLC on a C8-silica column. Fractions were collected and

assessed for presence of compounds cross-reacting with antibodies raised against either DmAMP1 or RsAFP2 using Elisa assays as described in Example 4.
As shown in figure 15. DmAMP1- CRPs eluted at a position identical or very close to that of authentic DmAMP1 in the line transformed with construct 3105 as well as in that transformed with construct 3106. Likewise, RsAFP2-CRPs were detected in both the construct 3105 and 3106 lines at an elution position identical or very close to that of authentic RsAFP2. None of the fractions reacted with both the anti-DmAMP1 and anti-RsAFP2 antibodies, indicating that an uncleaved fusion protein was not present in the extracts. No cross-reacting compounds were observed in a non-transformed line.

Thus it appears that the primary translation products of the transcription units of construct 3105 (IbAMP internal propeptide as linker peptide) and construct 3106 (partial DmAMP1 C-terminal propeptide with subtilisin-like protease site as a linker peptide) are somehow processed to yield separate DmAMP1-CRPs and RsAFP2-CRPs that appear to be identical or very closely related to DmAMP1 and RsAFP2, respectively, based on their chromatographic behavior.

<u>Example 7</u>

<u>Analysis of the subcellular location of coexpressed plant defensins</u>

In order to determine whether the coexpressed plant defensins are either secreted extracellularly or deposited intracellularly, extracellular fluid and intracellular extract fractions were obtained from leaves of homozygous transgenic Arabidopsis lines transformed with either constructs 3105 (line 2), 3106 (line 2) or 3108 (line 12). The cytosolic enzyme glucose-6-phosphate dehydrogenase was used as a marker to detect contamination of the extracellular fluid fraction with intracellular components. As shown in Table 2, glucose-6-phosphate dehydrogenase was partitioned in a ratio of about 80/20 between intracellular extract fractions and extracellular fluid fractions. In contrast, the majority of DmAMP1-CRP and RsAFP2-CRP content in all transgenic plants tested was found in the extracellular fluid fractions. These results indicate that both plant defensins released from the polyprotein precursors are deposited primarily in the apoplast. Hence, all processing steps that result in cleavage of the polyprotein structure must occur either in the apoplast or along the secretory pathway i.e. in

the endoplasmic reticulum, the Golgi apparatus or in vesicles trafficking between Golgi and apoplast.

Table 2: Relative abundance of glucose-6-phosphate dehydrogenase activity (GPD), DmAMP1 and RsAFP2 in the extracellular fluid (EF) and intracellular extract (IE) fractions obtained from transgenic Arabidopsis plants.

Construct	Relative abundance[1] (%) of					
	GPD		DmAMP1		RsAFP2	
	EF	IE	EF	IE	EF	IE
pFAJ3105	17	83	93	7	92	8
pFAJ3106	17	83	94	6	60	40
pFAJ3108	20	80	98	2	75	25

[1]Relative abundance is expressed as % of the sum of the contents in the EF and IE fractions.

Example 8

Purification of proteins processed from polyprotein precursor construct 3105

Transgenic line 14 from the population transformed with construct 3105 was further bred to obtain plants homozygous for the transgene. The DmAMP1-CRPs and RsAFP2-CRPs were purified by reversed phase chromatography from extracellular fluid prepared from leaves of this line. To this end, leaves were vacuum infiltrated with a buffer containing 50 mM MES (pH6) and a mixture of protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1mM N-ethylmaleimide, 5mM EDTA and 0.02 mM pepstatin A), and the extracellular fluid collected by centrifugation. Using this procedure homogenization and hence exposing DmAMP1-CRPs and RsAFP2-CRPs to compartimentalized proteases was avoided. The collected extracellular fluid was analyzed by RP-HPLC on a C8-silica column (Microsorb-MV, 4.6 x 250 mm, Rainin) and the fractions tested for presence of DmAMP1-CRPs and RsAFP2-CRPs by Elisa using antibodies raised against DmAMP1 and RsAFP2, respectively. The result of this analysis for the Arabidopsis transgenic line 14 transformed with construct 3105 is shown in figure 15. DmAMP1-CRPs eluted in two peaks, the latter of which eluted at a

position very close to that of authentic DmAMP1. RsAFP2-CRPs were found in a single peak that was well separated from the DmAMP1-CRP peaks and eluted at a position very close to that of authentic RsAFP2. None of the fractions reacted with both the anti-DmAMP1 and anti-RsAFP2 antibodies, indicating that an uncleaved fusion protein was absent from the extracellular fluid. Based on comparison of the peak areas of the DmAMP1-CRPs and RsAFP2-CRPs with those of a series of standards consisting of authentic Dm-AMP1 and RsAFP2, respectively, it was judged that the extract for the line transformed with construct 3105 contained about equal amounts of DmAMP1-CRPs and RsAFP2-CRPs. This indicates that cleavage of the polyprotein precursor in this line results in about equimolar amounts of DmAMP1-CRPs and RsAFP2-CRPs. Very similar chromatograms were obtained upon analysis of extracellular fluid prepared from transgenic line 2 (results not shown), indicating that the chromatographic pattern of DmAMP1-CRPs and RsAFP2-CRPs is independent from the transgenic line tested.

To test whether the purification procedure based on extracellular fluid preparation reflects the true composition in DmAMP-CRPs and RsAFP2-CRPs of the transgenic Arabidopsis leaves, an alternative purification procedure was developed starting from a crude leaf extract. To this end, leaves were homogenized under liquid nitrogen and extracted with 50 mM MES (pH6) containing a mixture of protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1mM N-ethylmaleimide, 5mM EDTA and 0.02 mM pepstatin A). The homogenate was cleared by centrifugation (10 min at 10000 x g). The supernatant was then fractionated by ion exchange chromatography (IEC) and subsequently by reversed phase chromatography (RPC). After each separation, fractions were collected and assessed for DmAMP-CRPs and RsAFP2-CRPs using two different Elisa assays with antibodies raised against DmAMP1 and RsAFP2, respectively. IEC was performed by passing the extract over a cation exchange column (Mono S, 5 x 50 mm, Pharmacia) at pH 6. When the column was eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM N-morpholino ethane sulfonic acid (MES) at pH 6, DmAMP1-CRPs were detected in fractions eluting between 0.17 and 0.33 M NaCl, while RsAFP2-CRPs eluted between 0.24 and 0.49 M NaCl. Fractions containing either DmAMP1-CRPs or RsAFP2-CRPs were pooled into two fractions (0.17 to 0.33 M NaCl; and 0.33 to 0.49 M NaCl) which were each subjected to RPC on a C8-silica column (Microsorb-MV, 4.6 x 250 mm, Rainin) eluted with a linear gradient

of acetonitrile (Figure 16). DmAMP1-CRPs eluted in two peaks, the latter of which eluted at a position very close to that of authentic DmAMP1. RsAFP2-CRPs were found in a single peak that was well separated from the DmAMP-CRP peaks and eluted at a position very close to that of authentic RsAFP2. Again, none of the fractions reacted with both the anti-DmAMP1 and anti-RsAFP2 antibodies, indicating that an uncleaved fusion protein was not present in the extracts.

The different DmAMP1-CRPs and RsAFP2-CRPs purified from extracellular fluid were subjected to N-terminal amino acid sequence analysis (procedures as described in Cammue *et al.*, 1992, J. Biol. Chem., 2228-2233) as well as to MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectrometry (Mann and Talbo, 1996, Curr. Opinion Biotechnol. 7, 11-19). The C-terminal amino acid was determined based on the best approximation of the predicted theoretical mass by the experimentally determined mass (Table 3). Both the minor DmAMP1-CRPs, p3105EF1, and the major DmAMP1-CRP, p3105EF2 (protein codes as in figure 15 and Table 3), had exactly the same N-terminal sequence as mature DmAMP1. p3105EF1 and p3105EF2 had masses that were consistent with the presence of a single additional serine residue at their C-terminal end compared to authentic DmAMP1. However, while the mass of p3105EF2 corresponded exactly (within experimental error) to that calculated for a DmAMP1 derivative with a C-terminal serine (hereafter called DmAMP1+S), that of p3105EF1 was in excess by about 8 dalton relative to the calculated mass for DmAMP1+S. Hence, this protein might be a DmAMP1+S derivative with reduced disulfide bridges. The RsAFP2-CRP fraction p3105EF3 represents, based on N-terminal sequence and mass data, an RsAFP2 derivative with the additional pentapeptide sequence DVEPG at its N-terminus. This protein is further referred to as DVEPG+RsAFP2. The different DmAMP1-CRPs and RsAFP2-CRPs purified from total leaf extract were analyzed in the same way. The analyses indicated that the same molecular species were present in the total leaf extract, i.e. DmAMP1+S, a putatively reduced form of DmAMP1+S, and DVEPG+RsAFP2 (Table 3 see Example 10 below).

The purified fractions containing the major processing products, DmAMP1+S and DVEPG+RsAFP2 respectively, were subjected to an antimicrobial activity test using the fungus *Fusarium culmorum* according to the procedure outlined by Cammue *et al.* (1992, J.

Biol. Chem. 267, 2228-2233). The specific antimicrobial activity, expressed as protein concentration required for 50 % growth inhibition of the test organism, of purified DmAMP1+S was identical to that of authentic DmAMP1. The specific antimicrobial activity of purified DVPEG−RsAFP2 was about 2-fold lower relative to that of authentic RsAFP2. The slight drop in specific antimicrobial activity of DVPEG+RsAFP2 is most likely due to the presence of 5 additional N-terminal amino acids. Nevertheless, our data prove that processing of the polyprotein precursors in transgenic plants can result in the release of bioactive proteins.

Analysis of the AFPs produced in transgenic plants transformed with construct 3105 reveals that the precursor is apparently processed by three cleavage steps (Figure 17):

(i) the precursor is cleaved at the C-terminal end of the leader peptide in the same way as for the authentic DmAMP1 precursor; (ii) the precursor is cleaved at the C-terminal end of the first amino acid of the linker peptide, thus releasing DmAMP1+S; (iii) the precursor is further processed at the N-terminal end of the fifth last residue of the linker peptide, thus releasing DVEPG+RsAFP2. It is not known which proteases effect the observed cleavages, nor how many different proteases are involved. Cleavages in the linker peptides might involve only endoproteinases or result from the coordinated action of endoproteinases and exopeptidases that further trim the cleavage products at their ends. Processing at the C-terminal side of the linker peptide occurs between the two acidic residues E and D. The acidic doublet might be a target sequence for a specific endoproteinase. An aspartic endoproteinase that is able to cleave between two consecutive acidic residues has previously been purified from Arabidopsis seeds (D'Hondt et al. 1993, J. Biol. Chem. 268, 20884-20891). It is worthwhile to mention that the sequence ED occurs at the very C-terminal end in five out of six internal propeptides of the IbAMP1 polyprotein precursor (Tailor et al. 1997, J. Biol. Chem. 272, 24480-24487). In one of the six internal IbAMP propeptides, more precisely the one that was used in construct 3105, the ED sequence does not occur at the C-terminal end of the propeptides but is separated by 4 amino acids from this end. Processing of this propeptide in *Impatiens balsamina* might involve cleavage of the ED sequence followed by partial N-terminal trimming of the resulting protein by an aminopeptidease.

It would be expected that an internal propeptide resembling the IbAMP1 propeptide used in construct 3105 but in which the ED dipeptidic sequence is moved to the C-terminal end of the propeptide, would result in a cleavage product with only one or no extra N-terminal amino acids in the protein located C-terminally from the internal propeptide. Alternatively, another IbAMP1 propeptide which already has an ED sequence at its C-terminal end (Tailor *et al.*, 1997, J. Biol. Chem. 272, 24480-24487) or a related sequence might give a similar improvement of processing accuracy.

Example 9

Purification of proteins processed from polyprotein precursor construct pFAJ3106

Transgenic line 9 from the population of *Arabidopsis* plants transformed with construct pFAJ3106 was further bred to obtain plants homozygous for the transgene. The DmAMP1-CRPs and RsAFP2-CRPs were purified by reversed phase chromatography from leaf extracellular fluid prepared in the same way as described above in Example 8 for the line transformed with construct pFAJ3105. The chromatogram of this separation is shown in Figure 18. DmAMP1-CRPs eluted in two peaks, called p3106EF1 and p3106EF2. Both fractions had the same N-terminal sequence as DmAMP1 (Table 3 see Example 10 below). The mass of p3106EF2 corresponded to that predicted for a DmAMP1 derivative with an additional lysine. We therefore conclude that it represents the cleavage product of the precursor cleaved at the signal peptide cleavage site and C-terminally behind the first residue (lysine) of the linker peptide; This protein is further referred to as DmAMP1+K.

The RsAFP2-CRP fraction was found by N-terminal amino acid sequencing to start by the sequence LIGKRQK. Hence, this protein, called QLIGKR+ RsAFP2, is derived from cleavage of the precursor N-terminally from the sixth last residue (glutamine) of the linker peptide. The proposed cleavage steps involved in processing of the precursor of construct pFAJ3106 are shown in Figure 17.

Example 10

Purification of proteins processed from polyprotein precursor construct pFAJ3108

Transgenic line 9 from the population of Arabidopsis plants transformed with construct pFAJ3108 was further bred to obtain plants homozygous for the transgene. The DmAMP1-

CRPs and RsAFP2-CRPs were purified from a total crude leaf extract of this line, following a procedure based on IEC and RPC as described above in Example 8 for the line transformed with construct 3105. The chromatograms of the IEC and RPC separations are shown in Figure 19. The IEC separation yielded two peaks containing DmAMP1-CRPs. However, no RsAFP2-CRPs could be detected in any of the eluate fractions. As RsAFP2-CRPs were clearly present in crude extracts and EF fractions of plants transformed with construct pFAJ3108 (see tables 1 and 2) the RsAFP2-CRPs must have been lost during the separation. The most likely explanation is that the RsAFP2-CRPs were not eluted from the IEC column with 0.5 M NaCl, the highest concentration used in the elution gradient. Fractions containing DmAMP1-CRPs were separated by RPC, yielding two DmAMP1-CRP peak. Analysis of this fraction by N-terminal sequencing and MALDI-TOF mass determination (Table 3) revealed that it represents a DmAMP1 derivative with an additional alanine at its C-terminus (DmAMP1+A). This protein results from cleavage of the precursor at the signal peptide cleavage site and C-terminally from the first residue (alanine) of the linker peptide (Figure 17).

Table 3: Mass determined by MALDI-TOF-MS or EI-MS and N-terminal sequence determined by automated Edman degradation of DmAMP1-CRP and RsAFP2-CRP fractions purified as described in Figures 15, 16, 18 and 19. Also shown are the predicted C-terminal sequence that gives best correspondence between experimental mass and theoretical mass.

Construct	Protein fraction (see Figures 15, 16, 18 and 19)	Mass determined by MALDI-TOF-MS	Mass determined by EI-MS	Determined N-terminal sequence (SEQ ID NOS 69-71	Predicted C-terminal sequence (SEQ ID NOS 72-76)	Theoretical mass for predicted sequence
pFAJ3105	p3105EF1	5614 $\pm$5	5608.3 $\pm$ 1	ELCEKAS	CYFNCS	5604.25
	p3105EF2	5602 $\pm$ 5	5604.9 $\pm$ 1	ELCEKAS	CYFNCS	5604.25
	p3105EF3	6223 $\pm$ 6	N.D.[1]	DVEPGQK	ICYFPC	6225.15
	p3105TE1	5610 $\pm$ 5	N.D.	ELCEKAS	CYFNPS	5604.25
	p3105TE2	5604 $\pm$ 5	N.D.	ELCEKAS	CYFNCS	5604.25
	p3105TE3	6224 $\pm$ 6	N.D.	DVEPGQK	ICYFPC	6225.15
pFAJ3106	p3106EF1	N.D.	N.D.	ELCEKAS	CYFNCK	5645.34
	p3106EF2	5640 $\pm$ 5	N.D.	ELCEKAS	CYFNCK	5645.34
	p3106EF3	N.D.	N.D.	LIGKRQK	ICYFPC	6295.38
pFAJ3108	p3108TE1	5583 $\pm$ 5	N.D.	ELCEKAS	CYFNCA	5588.25

[1]Not determined

Example 11

Modifications to construct pFAJ3105

From the analysis of Arabidopsis plants transformed with construct pFAJ3105 it is clear that the polyprotein precursor is indeed cleaved (see Table 3, Figure 17). However, cleavage occurs such that one amino acid from the linker peptide remains attached to the mature protein located N-terminally from the linker peptide, and that five amino acids remain attached to the mature protein located C-terminally from the linker peptide (see Figure 17). In order to reduce the number of linker peptide-derived amino acids attached to the mature proteins, which could possibly interfere with the functional properties of these mature proteins, a number of constructs have been designed in order to obtain cleavage occurring closer to (or even preferentially at) the borders of the mature proteins.

In construct pFAJ3343, the codon for the N-terminal residue of the linker peptide occurring in pFAJ3105 has been deleted. It is expected that cleavage of mature DmAMP1 will occur without addition of any amino acid from the linker peptide (Figure 20). In constructs pFAJ3344, pFAJ3345 and pFAJ3346, the codons at the carboxyl-terminal end of the linker peptide in pFAJ3105 have been modified such that the last two, four and five residues have been deleted, respectively. It is expected that the number of residues remaining attached to the N-terminal end of RsAFP2 after cleavage will be respectively three, one and zero in constructs pFAJ3344, pFAJ3345 and pFAJ3346 (Figure 20). Other constructs can be made in which the number of residues at either the N- or C-terminal end of the linker peptide region in construct pFAJ3105 is reduced.

In construct pFAJ3105 the linker peptide is derived from the fourth internal propeptide of the IbAMP precursor (Tailor R.H. *et al.*, 1997, J. Biol. Chem. 272, 24480-24487). In construct pFAJ3369, this linker peptide has been replaced by the first internal propeptide of the IbAMP precursor (Tailor R.H. *et al.*, 1997, ibid.). In the latter linker peptide the doublet of acidic residues occurs at the C-terminus. It is expected that the cleavage will occur such that only one residue will remain attached to the N-terminus of RsAFP2 (Figure 20).

Example 12

Construction of a construct for expression of a polyprotein with four mature protein domains

The polyprotein region in construct pFAJ3367 consists of the signal peptide region of DmAMP1 cDNA followed by the coding regions of four different antimicrobial peptides, each separated by the first internal propeptide region of the IbAMP precursor. The coding region for the four different antimicrobial proteins are, in order (see Figure 21):

1. The plant defensin DmAMP1 (Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262)
2. The plant defensin RsAFP2 (Terras F.R.G. *et al.*, 1995, Plant Cell 7, 573-588)
3. The plant defensin HsAFP1 (Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262)
4. The lipid transfer protein-like protein AceAMP1 (Cammue B.P.A. *et al.*, 1995, Plant Physiol. 109, 445-455)

This construct will give rise to four different mature antimicrobial proteins (DmAMP1, RsAFP2, HsAFP1 and AceAMP1), each of which secreted to the extracellular space.

Other constructs can be made other mature peptide regions and with any other linker peptide regions described above.

Example 13

Modifications to constructs pFAJ3106, pFAJ 3107 and pFAJ 3108

The polyprotein encoded by constructs pFAJ3106, pFAJ3107 and pFAJ3108 contain linker peptides with the Kex2 recognition site IGKR at their C-terminal ends. Jiang L. and Rogers J.C. (1999, Plant J. 18, 23-32) have shown that polyproteins containing a IGKR site are not or poorly cleaved in transgenic tobacco plants. Improved cleavage was observed in polyproteins in which the IGKR sequence was replaced by the IGKRIGKRIGKR (SEQ ID NO 77) sequence.

Constructs pFAJ3106-2, pFAJ3107-2 and pFAJ3108-2 are identical to constructs pFAJ3106, pFAJ3107 and pFAJ3108 except for the replacement of the IGKR coding region by a region coding for IGKRIGKRIGKR (Figure 22). Polyproteins encoded by these constructs will be efficiently cleaved both at the N-terminal end and the C-terminal end of the linker peptide.

Other constructs can be made in which the number of residues at either the N- or C-terminal end of the linker peptide region in constructs pFAJ3106, pFAJ3107 or pFAJ3108 is reduced.

Example 14

Polyprotein constructs based on hybrid linker peptides containing the 2A sequence

The foot-and-mouth disease virus (FMDV) RNA is translated as a polyprotein whose cleavage depends on a 20 amino acids sequence called the 2A sequence (Ryan and Drew 1994, EMBO J. 13, 928-933). Cleavage of the polyproteins joined by the 2A sequence occurs between the 19th amino acid (G) and the 20th amino acid (P) of the 2A sequence via a process which is apparently independent of processing enzymes and which might be due to improper formation of the peptide bond between G and P (Halpin *et al.*, 1999, Plant J. 17, 453-459). Halpin C. *et al.* 1999 (Plant J. 17, 453-459) have shown that polyproteins containing the FMDV 2A sequence as a linker peptide are efficiently cleaved when expressed in plants. One major drawback of the use of the FMDV 2A sequence as a linker peptide, however, is that cleavage does not occur at the N-terminus of the linker peptide. Hence, a relatively long stretch of 19 amino acids corresponding to the first 19 residues of the FMDV 2A sequence remains attached to the C-terminus of the mature protein. This additional stretch of 19 residues may interfere with the functional properties of the protein to which it is attached.

In order to address this problem of incomplete removal of the linker peptide after cleavage, hybrid linker peptides consisting at their N-terminal part of a linker peptide described in constructs pFAJ3105, pFAJ3106, pFAJ3107 or pFAJ3108 (or a part of such peptide) and at their C-terminal part of the FMDV 2A sequence (or a part of such peptide) are proposed. Examples of constructs based on this principle are constructs pFAJ3370 and pFAJ3368 (Figure 23). Construct pFAJ3370 has a polyprotein region identical to that of construct pFAJ3105 except that the linker peptide is a 29 amino acids peptide consisting of the first 9 amino acids of the fourth internal propeptide of the IbAMP precursor (Tailor R.H. *et al.*, 1997, J. Biol. Chem. 272, 24480-24487) followed by the 20 amino acids of the entire FMDV 2A sequence. Cleavage of this linker peptide should release a mature DmAMP1 with an additional serine at its C-terminus and a mature RsAFP2 with an additional proline at its N-terminus.

Construct pFAJ3368 is identical to construct pFAJ3370 except that the C-terminal mature protein domain (in this case encoding RsAFP2) is replaced by a domain encoding this

mature protein domain preceded by a signal peptide domain (in this case encoding RsAFP2 with its own signal peptide). If cleavage between G and P of the FMDV 2A sequence occurs prior to full translocation of the polyprotein into the endoplasmic reticulum then it is expected that construct pFAJ3368 will provide better targetting of both mature proteins to the extracellular space in comparison to construct pFAJ3370. In this case, the secreted mature proteins will consist of DmAMP1 with an additional serine at its C-terminus and RsAFP2 with no added amino acids. If cleavage between G and P of the FMDV 2A sequence occurs after translocation of the polyprotein into the endoplasmic reticulum, then it is expected that the signal peptide attached to RsAFP2 will not be efficiently removed and in this case construct pFAJ3370 will be preferred over pFAJ3368.

CLAIMS

1. A method of improving expression levels of one or more proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide, said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

2. A method according to claim 1 wherein said promoter region is operably linked to a signal sequence, said signal sequence being operably linked to the said two or more protein encoding regions and a 3'-terminator region.

3. A method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

4. A method according to any of the preceding claims wherein at least 40% of the sequence of said linker propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine.

5. A method according to any of the preceding claims wherein said linker propeptide has within 7 residues of its N- or C- terminal cleavage site a sequence with two to five

consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

6. A method according to any of the preceding claims wherein the DNA sequence encoding said linker propeptide encodes a propeptide isolatable from a plant protein, or a virus or a variant thereof or a fragment of either of these which provides a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

7. A method according to any of the preceding claims wherein the DNA sequence encoding said linker propeptide encodes a propeptide isolatable from a plant protein or a fragment thereof.

8. A method according to claim 6 or claim 7 wherein the DNA sequence encoding said linker propeptide encodes a chimeric propeptide comprising a propeptide isolatable from one or more plant proteins and/or a virus, or a variant thereof or a fragment of either of these.

9. A method according to any one of claim 7 or claim 8 wherein the plant protein is a precursor of a plant defensin, or a hevein-type antimicrobial protein .

10. A method according to claim 9 wherein the plant protein is an antimicrobial protein derived from the genus Impatiens.

11. A method according to claim 10 wherein the propeptide comprises SEQ ID NO. 3, 29, 21, 22, 23 or 24.

12. A method according to claim 8 wherein the propeptide comprises a C-terminal propeptide from Dm-AMP1 or Ac-AMP2 or a fragment thereof, or a variant of any of these.

13. A method according to claim 12 wherein the propeptide comprises SEQ ID NO. 4, 6, 7, 25, 26 or 27.

14. A method according to any one of the preceding claims wherein the propeptide is a chimeric propeptide.

15. A method according to any one of claim 13 wherein the chimeric propeptide comprises a virus propeptide or a fragment thereof, and a propeptide isolated from a plant protein or a fragment thereof.

16. A method according to claim 15 wherein the virus is a picornovirus.

17. A method according to claim 15 or 16 wherein the chimeric propeptide comprises SEQ ID NO 28 as the virus propeptide sequence.

18. A method according to any of the preceeding claims wherein the linker propeptide has a protease processing site engineered at either or both ends thereof.

19. A method according to claim 18 wherein the protease processing site is a subtilisin - like protease processing site.

20. A method according to claim 2 or 3 wherein the signal sequence is derived from a plant defensin gene.

21. A method according to any of the preceding claims wherein one or more of the multiple proteins is a defense protein.

22. Use of a propeptide cleavable in the secretory pathway of a plant linker for a polyprotein precursor synthesized in a transgenic plant.

23. Use of a propeptide according to claim 22 wherein the propeptide is derived from a plant protein or from a virus.

24. Use of a propeptide according to claim 22 or claim 23 wherein the propeptide is derived from a plant protein and the protein is a precursor of a plant defensin, or a hevein-type antimicrobial protein or is isolatable from the genus Impatiens.

25. Use of a propeptide as a cleavable linker in polyprotein precursors synthesized via the secretory pathway in transgenic plants wherein said propeptide linker is as defined in claim 4 or claim 5.

26. Use of a propeptide sequence rich in the small amino acids A, V, S and T and containing dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue as a cleavable linker sequence wherein said sequence is isolatable from a plant defensin or a hevein-type antimicrobial peptide.

27. A DNA construct comprising a DNA sequence comprising a promoter region operably linked to a plant derived signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3' terminator-region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a post-translational cleavage site.

28. A DNA construct comprising a DNA sequence comprising a promoter region operably linked to two or more protein encoding regions and a 3' terminator-region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide encoding a C-terminal propeptide from the Dm-AMP gene or from the Ac-AMP gene, said propeptide providing a post-translational cleavage site

29. A DNA construct according to claim 27 or claim 28 wherein the DNA sequence encoding the linker propeptide additionally comprises one or more protease recognition sites at either or both ends thereof.

30. A vector comprising a DNA construct according to any of claims 19 to 21.

31. A transgenic plant transformed with a DNA construct or a vector according to any one of claims 27 to 30.

32. Use of a DNA construct comprising a DNA sequence comprising a promoter region operably linked to two or more protein encoding regions and a 3' terminator region wherein said promoter encoding region are separated from each other by a DNA sequence coding for a linker propeptide, said propeptide providing a post-translational cleavage site for increasing protein expression levels in a transgenic plant. or a vector comprising said construct, for increasing protein expression levels in a transgenic plant.

33. A nucleic acid which encodes a peptide of SEQ ID NO 4, 6, 7, 29, 21, 22, 23, 24, 25, 26, 27, 28 or the linker peptide shown in Figure 34 or a variant of any of these.

34. A nucleic acid according to claim 33 which encodes a peptide of SEQ ID NO 4, 6, 7, 29, 21, 22, 23, 24, 25, 26, 27, 28 or the linker peptide shown in Figure 34.

35. A nucleic acid according to claim 33 which encodes a peptide comprising SEQ ID NO 77 linked at the C-terminal end of SEQ ID NO 4, 6, 7, 29, 21, 22, 23, 24, 25, 26, 27, 28 or the linker peptide shown in Figure 34

Fig.1.

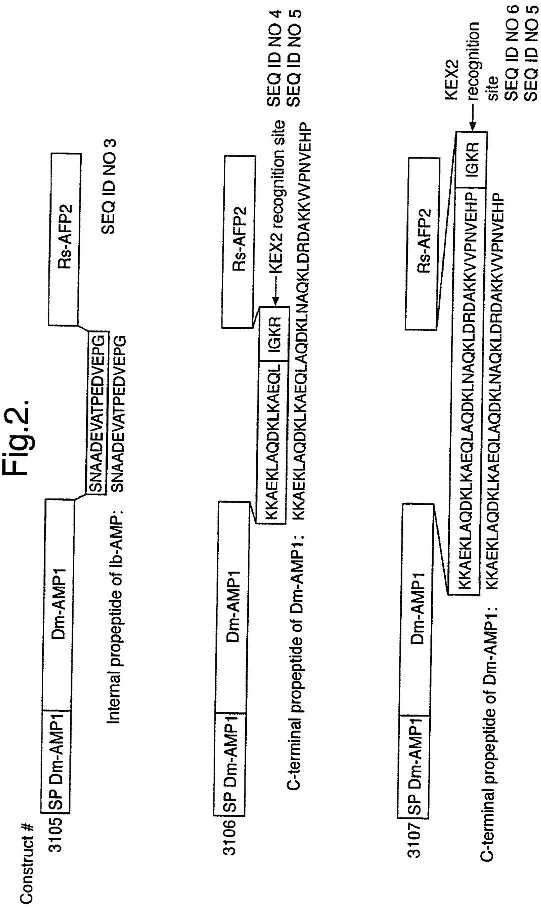
```
     M   V   N   R   S   V   A   F   S   A   F   V   L   I   L   F   V   L   A   I
1    ATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCCATC

     S
61   TCAGGTTATCAAATCTTTAGTTCATTTATTGAATATGATAGTATTTATATTCTTTTATGG
                                           intron
                                           D   I   A   S   V   S   G   E
121  TTTTATGTGTTCTGACAAGTTGCAAATATTGAGTAGATATCGCATCCGTTAGTGGAGAAC

     L   C   E   K   A   S   K   T   W   S   G   N   C   G   N   T   G   H   C   D
181  TATGCGAGAAAGCTAGCAAGACATGGTCGGGAAACTGTGGCAATACGGGACATTGTGACA
                                    NcoI
     N   Q   C   K   S   W   E   G   A   A   H   G   A   C   H   V   R   N   G   K
241  ACCAATGTAAATCATGGGAGGGTGCGGCCCATGGAGCGTGTCATGTGCGTAACGGGAAAC
                                                 HindIII
     Q   M   C   F   C   Y   F   N   C   K   K   A   E   K   L   A   Q   D   K   L
301  AGATGTGTTTCTGTTACTTCAATTGTAAAAAAGCCGAAAAGCTTGCTCAAGACAAACTTA
                                                 HindIII
     K   A   E   Q   L   A   Q   D   K   L   N   A   Q   K   L   D   R   D   A   K
361  AAGCCGAACAACTCGCTCAAGACAAACTTAATGCCCAAAAGCTTGACCGTGATGCCAAGA

     K   V   V   P   N   V   E   H   P                SEQ ID NO 2
421  AAGTGGTTCCAAACGTTGAACATCCG                       SEQ ID NO 1
```

Fig.2.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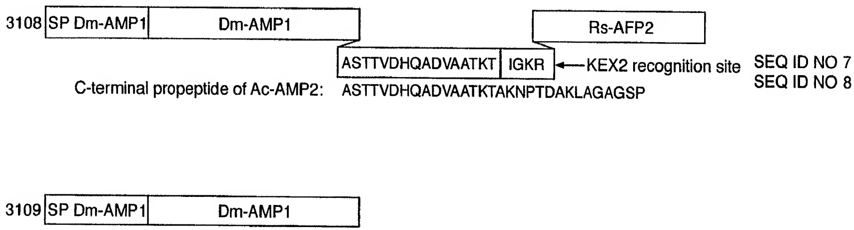
Dm-AMP1
SNAADEVATPEDVEPG
Rs-AFP2
Internal propeptide of Ib-AMP: SNAADEVATPEDVEPG
SEQ ID NO 3
3106
SP Dm-AMP1
Dm-AMP1
KKAEKLAQDKLKAEQL
IGKR
Rs-AFP2
KEX2 recognition site
C-terminal propeptide of Dm-AMP1: KKAEKLAQDKLKAEQLAQDKLNAQKLDRDAKKVVPNVEHP
SEQ ID NO 4
SEQ ID NO 5
3107
SP Dm-AMP1
Dm-AMP1
KKAEKLAQDKLKAEQLAQDKLNAQKLDRDAKKVVPNVEHP
IGKR
Rs-AFP2
KEX2 recognition site
C-terminal propeptide of Dm-AMP1: KKAEKLAQDKLKAEQLAQDKLNAQKLDRDAKKVVPNVEHP
SEQ ID NO 6
SEQ ID NO 5

Fig.2 (Cont).


3108
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Dm-AMP1
Rs-AFP2
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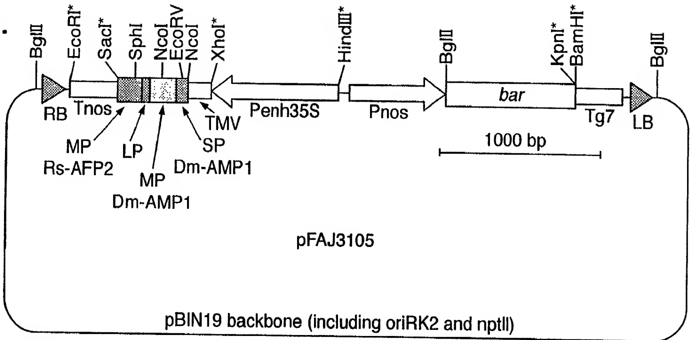
IGKR
KEX2 recognition site
SEQ ID NO 7
C-terminal propeptide of Ac-AMP2:
ASTTVDHQADVAATKTAKNPTDAKLAGAGSP
SEQ ID NO 8
3109
SP Dm-AMP1
Dm-AMP1

Fig.3.

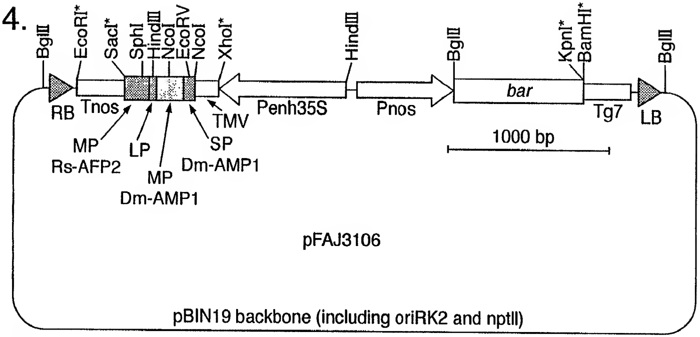


Symbols

RB: right border of T-DNA
Tnos: terminator of T-DNA nopaline synthase gene
MP Rs-AFP2: mature protein domain of Rs-AFP2
LP: Ib-AMP internal propeptide
MP Dm-AMP1: mature protein domain of Dm-AMP1 cDNA
SP Dm-AMP1: signal peptide domain of Dm-AMP1 cDNA
TMV: tobacco mosaic virus 5' leader sequence
Penh35S: promotor of 35S RNA of cauliflower mosaic virus with duplicated enhancer region
Pnos: promotor of T-DNA nopaline synthase gene
bar: basta resistance encoding gene
Tg7: terminator of T-DNA gene 7
LB: left border of T-DNA

*: unique restriction site

Fig.4.

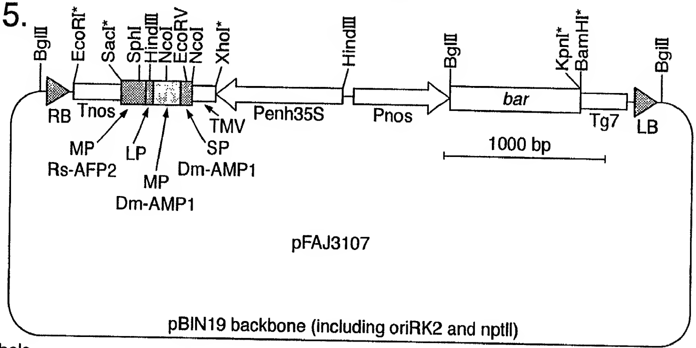


Symbols

RB: right border of T-DNA
Tnos: terminator of T-DNA nopaline synthase gene
MP Rs-AFP2: mature protein domain of Rs-AFP2
LP: first 16 AA of Dm-AMP1 C-terminal propeptide and subtilisin-like protease recognition site IGKR
MP Dm-AMP1: mature protein domain of Dm-AMP1 cDNA
SP Dm-AMP1: signal peptide domain of Dm-AMP1 cDNA
TMV: tobacco mosaic virus 5' leader sequence
Penh35S: promotor of 35S RNA of cauliflower mosaic virus with duplicated enhancer region
Pnos: promotor of T-DNA nopaline synthase gene
bar: basta resistance encoding gene
Tg7: terminator of T-DNA gene 7
LB: left border of T-DNA

*: unique restriction site

Fig.5.

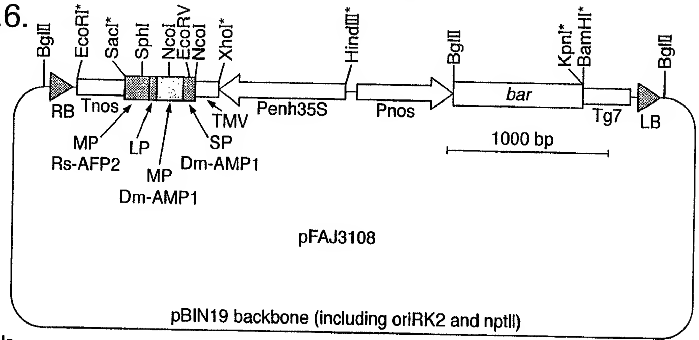


Symbols

RB: right border of T-DNA
Tnos: terminator of T-DNA nopaline synthase gene
MP Rs-AFP2: mature protein domain of Rs-AFP2
LP: Dm-AMP1 C-terminal propeptide domain and subtilisin-like protease recognition site IGKR
MP Dm-AMP1: mature protein domain of Dm-AMP1 cDNA
SP Dm-AMP1: signal peptide domain of Dm-AMP1 cDNA
TMV: tobacco mosaic virus 5' leader sequence
Penh35S: promotor of 35S RNA of cauliflower mosaic virus with duplicated enhancer region
Pnos: promotor of T-DNA nopaline synthase gene
bar: basta resistance encoding gene
Tg7: terminator of T-DNA gene 7
LB: left border of T-DNA

*: unique restriction site

Fig.6.

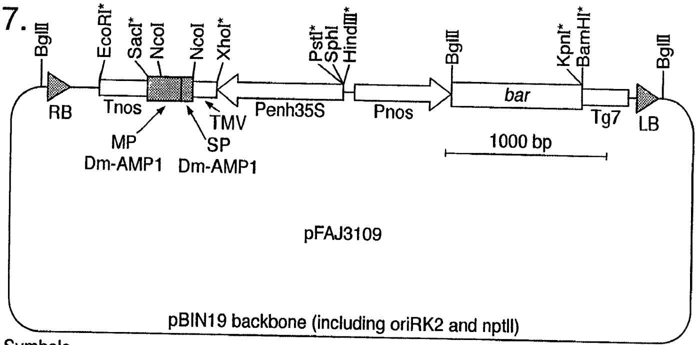


Symbols

RB: right border of T-DNA
Tnos: terminator of T-DNA nopaline synthase gene
MP Rs-AFP2: mature protein domain of Rs-AFP2
LP: first 16 AA of Ac-AMP2 C-terminal propeptide domain and subtilisin-like protease recognition site IGKR
MP Dm-AMP1: mature protein domain of Dm-AMP1 cDNA
SP Dm-AMP1: signal peptide domain of Dm-AMP1 cDNA
TMV: tobacco mosaic virus 5' leader sequence
Penh35S: promotor of 35S RNA of cauliflower mosaic virus with duplicated enhancer region
Pnos: promotor of T-DNA nopaline synthase gene
bar: basta resistance encoding gene
Tg7: terminator of T-DNA gene 7
LB: left border of T-DNA

*: unique restriction site

Fig.7.



Symbols

RB: right border of T-DNA
Tnos: terminator of T-DNA nopaline synthase gene
MP Dm-AMP1: mature protein domain of Dm-AMP1
SP Dm-AMP1: signal peptide domain of Dm-AMP1 cDNA
TMV: tobacco mosaic virus 5' leader sequence
Penh35S: promotor of 35S RNA of cauliflower mosaic virus with duplicated enhancer region
Pnos: promotor of T-DNA nopaline synthase gene
bar: basta resistance encoding gene
Tg7: terminator of T-DNA gene 7
LB: left border of T-DNA

*: unique restriction site

Fig.8.

pFAJ3105

```
 XhoI
CTCGAGTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACT

            NcoI
ATTTACAATTACACCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTT
               M  V  N  R  S  V  A  F  S  A  F  V  L  I  L

TTCGTGCTCGCCATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGC
 F  V  L  A  I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S

AAGACGTGGTCGGGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGG
 K  T  W  S  G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W

GAGGGTGCGGCCCATGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTAC
 E  G  A  A  H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y

TTCAATTGTTCCAACGCTGCTGACGAGGTGGCTACCCCAGAGGACGTGGAGCCAGGACAG
 F  N  C  S  N  A  A  D  E  V  A  T  P  E  D  V  E  P  G  Q

AAGTTGTGCCAAAGGCCAAGTGGGACATGGTCAGGAGTCTGTGGAAACAATAACGCATGC
 K  L  C  Q  R  P  S  G  T  W  S  G  V  C  G  N  N  N  A  C

AAGAATCAGTGCATTAGACTTGAGAAAGCACGACATGGATCTTGCAACTATGTCTTCCCA
 K  N  Q  C  I  R  L  E  K  A  R  H  G  S  C  N  Y  V  F  P

                                          SacI
GCTCACAAGTGTATCTGCTACTTTCCTTGTTAATAGGAGCTC    SEQ ID NO 9
 A  H  K  C  I  C  Y  F  P  C  -  -           SEQ ID NO 10
```

Fig.9. pFAJ3106

```
XhoI
CTCGAGTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACT

                NcoI
ATTTACAATTACACCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTT
                 M  V  N  R  S  V  A  F  S  A  F  V  L  I  L

TTCGTGCTCGCCATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGC
 F  V  L  A  I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S

AAGACGTGGTCGGGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGG
 K  T  W  S  G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W

GAGGGTGCGGCCCATGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTAC
 E  G  A  A  H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y

TTCAATTGTAAAAAAGCCGAAAAGCTTGCTCAAGACAAACTTAAAGCCGAACAACTCATC
 F  N  C  K  K  A  E  K  L  A  Q  D  K  L  K  A  E  Q  L  I

GGAAAGAGGCAGAAGTTGTGCCAAAGGCCAAGTGGGACATGGTCAGGAGTCTGTGGAAAC
 G  K  R  Q  K  L  C  Q  R  P  S  G  T  W  S  G  V  C  G  N

AATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAAGCACGACATGGATCTTGCAAC
 N  N  A  C  K  N  Q  C  I  R  L  E  K  A  R  H  G  S  C  N

                                                   SacI
TATGTCTTCCCAGCTCACAAGTGTATCTGCTACTTTCCTTGTTAATAGGAGCTC      SEQ ID NO 11
 Y  V  F  P  A  H  K  C  I  C  Y  F  P  C  -  -             SEQ ID NO 12
```

Fig.10.

pFAJ3107

```
XhoI
CTCGAGTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACT

                NcoI
ATTTACAATTACACCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTT
               M  V  N  R  S  V  A  F  S  A  F  V  L  I  L

TTCGTGCTCGCCATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGC
 F  V  L  A  I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S

AAGACGTGGTCGGGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGG
 K  T  W  S  G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W

GAGGGTGCGGCCCATGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTAC
 E  G  A  A  H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y

TTCAATTGTAAAAAAGCCGAAAAGCTTGCTCAAGACAAACTTAAAGCCGAACAACTCGCT
 F  N  C  K  K  A  E  K  L  A  Q  D  K  L  K  A  E  Q  L  A

CAAGACAAACTTAATGCCCAAAAGCTTGACCGTGATGCCAAGAAAGTGGTTCCAAACGTT
 Q  D  K  L  N  A  Q  K  L  D  R  D  A  K  K  V  V  P  N  V

GAACATCCGATCGGAAAGAGGCAGAAGTTGTGCCAAAGGCCAAGTGGGACATGGTCAGGA
 E  H  P  I  G  K  R  Q  K  L  C  Q  R  P  S  G  T  W  S  G

GTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAAGCACGACAT
 V  C  G  N  N  N  A  C  K  N  Q  C  I  R  L  E  K  A  R  H

GGATCTTGCAACTATGTCTTCCCAGCTCACAAGTGTATCTGCTACTTTCCTTGTTAATAG
 G  S  C  N  Y  V  F  P  A  H  K  C  I  C  Y  F  P  C  -  -

 SacI
GAGCTC
```

SEQ ID NO 15
SEQ ID NO 16

Fig.11. pFAJ3108

```
  XhoI
CTCGAGTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACT

                NcoI
ATTTACAATTACACCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTT
                 M  V  N  R  S  V  A  F  S  A  F  V  L  I  L

TTCGTGCTCGCCATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGC
 F  V  L  A  I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S

AAGACGTGGTCGGGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGG
 K  T  W  S  G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W

GAGGGTGCGGCCCATGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTAC
 E  G  A  A  H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y

TTCAATTGTGCCAGTACTACTGTGGATCACCAAGCTGATGTTGCTGCCACCAAAACTATC
 F  N  C  A  S  T  T  V  D  H  Q  A  D  V  A  A  T  K  T  I

GGAAAGAGGCAGAAGTTGTGCCAAAGGCCAAGTGGGACATGGTCAGGAGTCTGTGGAAAC
 G  K  R  Q  K  L  C  Q  R  P  S  G  T  W  S  G  V  C  G  N

AATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAAGCACGACATGGATCTTGCAAC
 N  N  A  C  K  N  Q  C  I  R  L  E  K  A  R  H  G  S  C  N

                                                 SacI
TATGTCTTCCCAGCTCACAAGTGTATCTGCTACTTTCCTTGTTAATAGGAGCTC
 Y  V  F  P  A  H  K  C  I  C  Y  F  P  C  -  -          SEQ ID NO 17
                                                         SEQ ID NO 18
```

Fig.12.

pFAJ3109

XhoI
CTCGAGTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACT

NcoI
ATTTACAATTACACCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTT
M V N R S V A F S A F V L I L

TTCGTGCTCGCCATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGC
F V L A I S D I A S V S G E L C E K A S

AAGACGTGGTCGGGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGG
K T W S G N C G N T G H C D N Q C K S W

GAGGGTGCGGCCCATGGAGCGTGTCATGTGCGTAATGGGAAACACATGTGTTTCTGTTAC
E G A A H G A C H V R N G K H M C F C Y

SacI
TTCAATTGTTGAGCTC
F N C -

SEQ ID NO 19
SEQ ID NO 20

Fig.13.

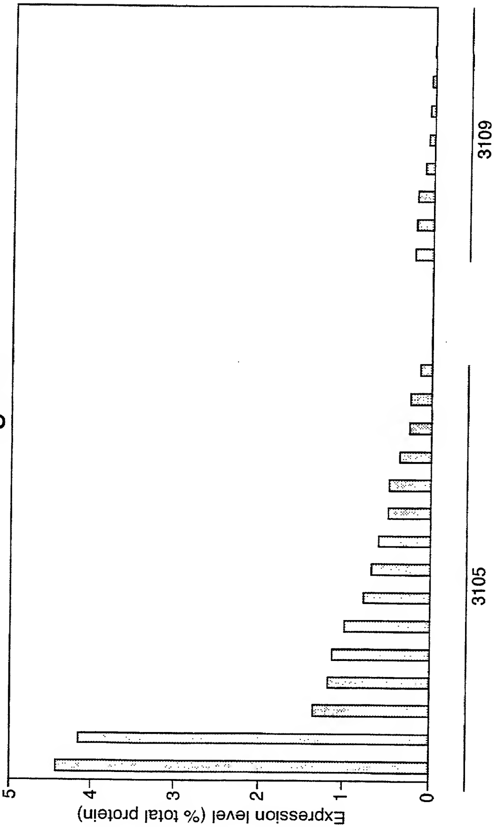


Fig.14A.

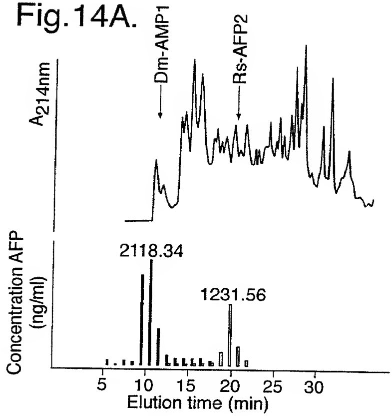


Fig.14B.

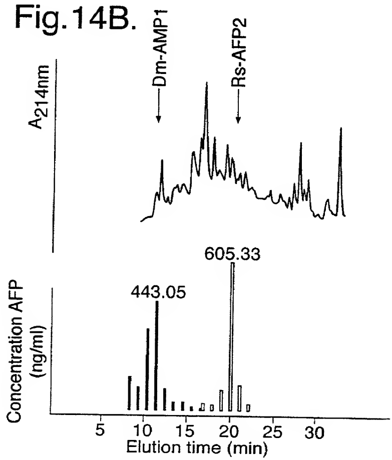


Fig.15.

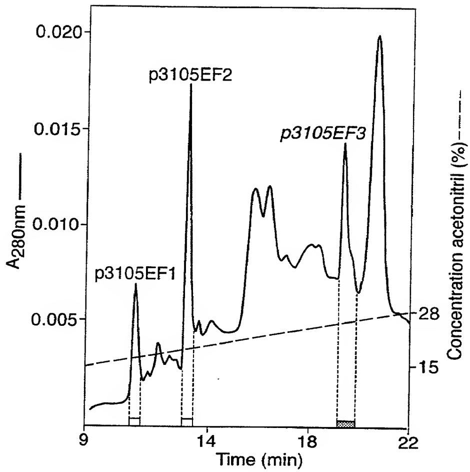


Fig.17.

Construct	Sequence
pFAJ3105	*MVN* ..... *VSG*ELC .... FNCSNAADEVATPEDVEPGQKL... FPC
pFAJ3106	*MVN* ..... *VSG*ELC .... FNCKKAEKLAQDKLKAEQLIGKRQKL... FPC
pFAJ3108	*MVN* ..... *VSG*ELC .... FNCASTTVDHQADVAATKTIGKRQKL... FPC

Fig.16.

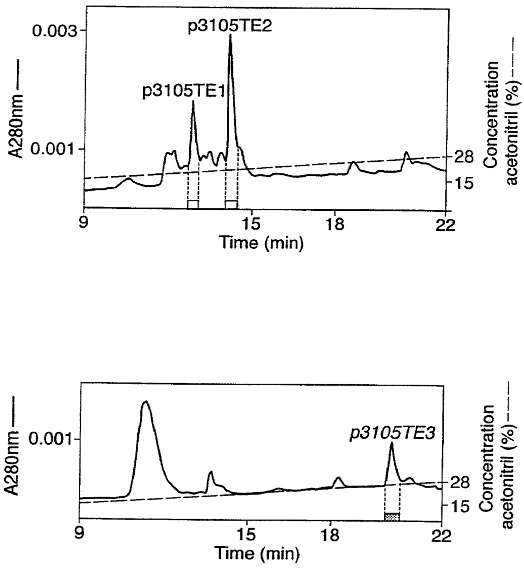

0.003
0.001
A280nm
p3105TE2
p3105TE1
Concentration acetonitril (%)
28
15
9
15
18
22
Time (min)
0.001
A280nm
p3105TE3
Concentration acetonitril (%)
28
15
9
15
18
22
Time (min)

Fig.18.

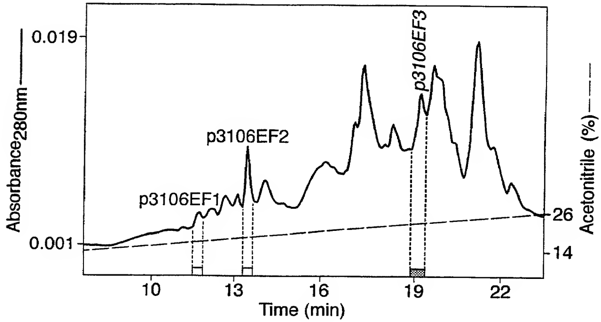

p3106EF3
p3106EF2
p3106EF1
0.019
0.001
Absorbance280nm
Acetonitrile (%)
26
14
10
13
16
19
22
Time (min)

Fig.19.

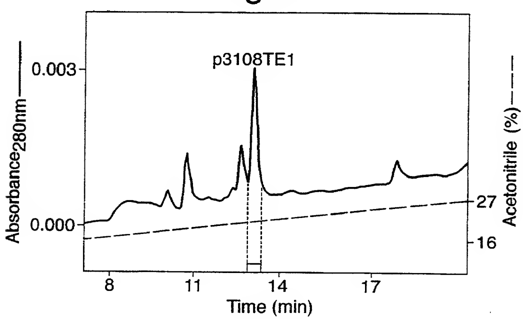

p3108TE1
0.003
0.000
Absorbance280nm
Acetonitrile (%)
27
16
8
11
14
17
Time (min)

Fig.20.

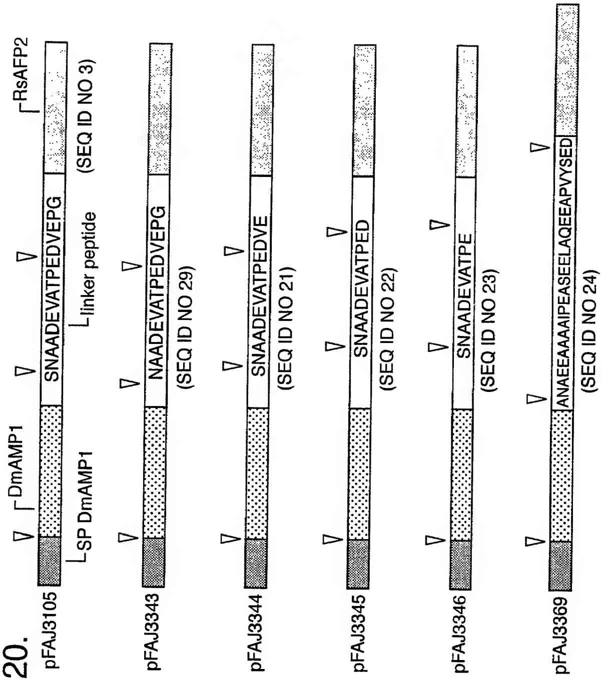

DmAMP1
RsAFP2
SP DmAMP1
linker peptide
pFAJ3105
SNAADEVATPEDVEPG
(SEQ ID NO 3)
pFAJ3343
NAADEVATPEDVEPG
(SEQ ID NO 29)
pFAJ3344
SNAADEVATPEDVE
(SEQ ID NO 21)
pFAJ3345
SNAADEVATPED
(SEQ ID NO 22)
pFAJ3346
SNAADEVATPE
(SEQ ID NO 23)
pFAJ3369
ANAEEAAAIPEASEELAQEEAPVYSED
(SEQ ID NO 24)

Fig.21.

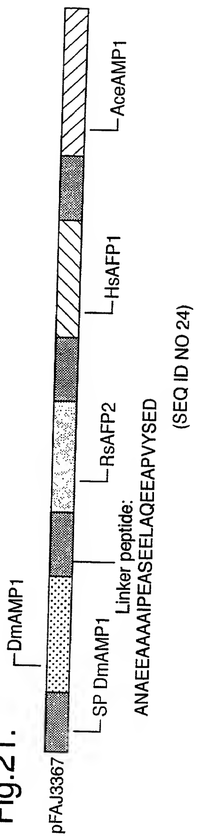

pFAJ3367
SP DmAMP1
DmAMP1
Linker peptide:
ANAEEAAAAIPEASEELAQEEAPVYSED
RsAFP2
HsAFP1
AceAMP1
(SEQ ID NO 24)

Fig.23.

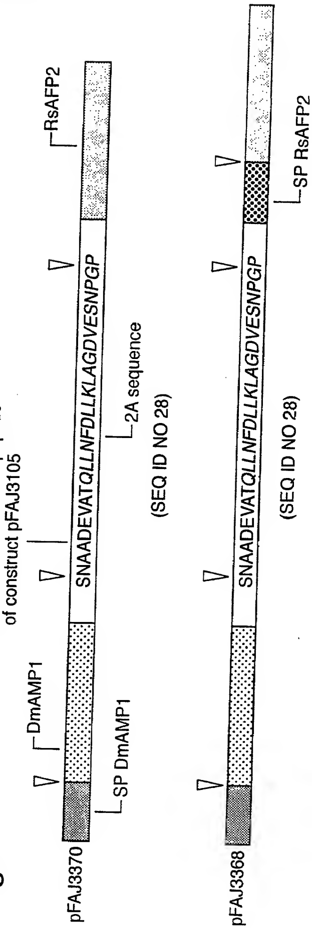

pFAJ3370
SP DmAMP1
DmAMP1
Aminoterminus of the linker peptide
of construct pFAJ3105
SNAADEVATQLLNFDLLKLAGDVESNPGP
2A sequence
(SEQ ID NO 28)
RsAFP2
pFAJ3368
SNAADEVATQLLNFDLLKLAGDVESNPGP
(SEQ ID NO 28)
SP RsAFP2

Fig.22.

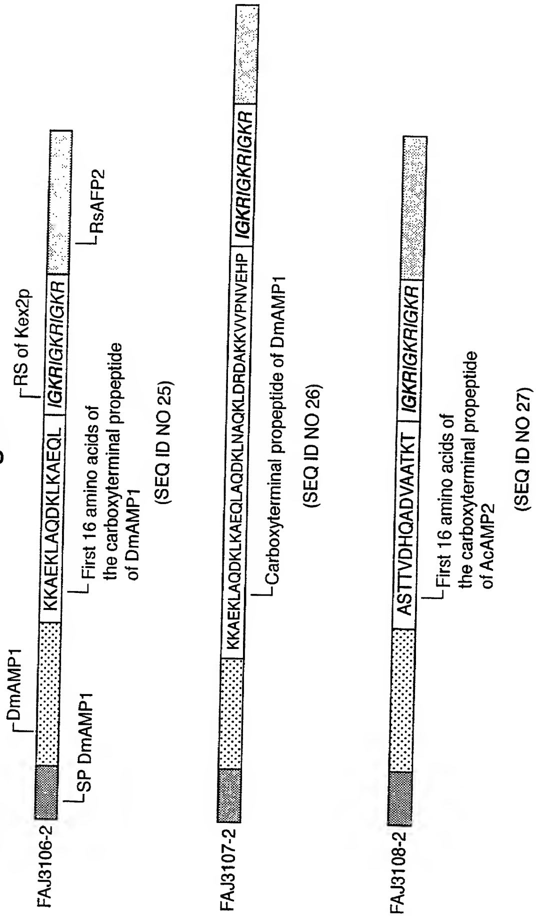

FAJ3106-2
DmAMP1
SP DmAMP1
KKAEKLAQDKLKAEQL
First 16 amino acids of the carboxyterminal propeptide of DmAMP1
IGKRIGKRIGKR
RS of Kex2p
RsAFP2
(SEQ ID NO 25)
FAJ3107-2
KKAEKLAQDKLKAEQLAQDKLNAQKLDRDAKKVVPNVEHP
Carboxyterminal propeptide of DmAMP1
IGKRIGKRIGKR
(SEQ ID NO 26)
FAJ3108-2
ASTTVDHQADVAATKT
First 16 amino acids of the carboxyterminal propeptide of AcAMP2
IGKRIGKRIGKR
(SEQ ID NO 27)

Fig.24.

pFAJ3343

```
 NcoI
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
   M  V  N  R  S  V  A  F  S  A  F  V  L  I  L  F  V  L  A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
 I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S  K  T  W  S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
 G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W  E  G  A  A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTAAC
 H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y  F  N  C  N

GCGGCCGACGAGGTGGCTACCCCAGAGGACGTGGAACCTGGTCAGAAGTTGTGCCAAAGG
 A  A  D  E  V  A  T  P  E  D  V  E  P  G  Q  K  L  C  Q  R

CCAAGTCGTACATGGTCAGGAGTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATT
 P  S  R  T  W  S  G  V  C  G  N  N  N  A  C  K  N  Q  C  I

AGACTTGAGAAAGCACGACATGGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATC
 R  L  E  K  A  R  H  G  S  C  N  Y  R  F  P  A  H  K  C  I
                      SacI
TGCTACTTTCCTTGTTAATAGGAGCTC             (SEQ ID NO 30)
 C  Y  F  P  C  -  -                    (SEQ ID NO 31)
```

pFAJ3344

Fig.25.

NcoI

```
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
   M  V  N  R  S  V  A  F  S  A  F  V  L  I  L  F  V  L  A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
 I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S  K  T  W  S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
 G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W  E  G  A  A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTTCC
 H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y  F  N  C  S

AACGCGGCCGACGAGGTGGCTACCCCAGAGGACGTGGAACAGAAGTTGTGCCAAAGGCCA
 N  A  A  D  E  V  A  T  P  E  D  V  E  Q  K  L  C  Q  R  P

AGTCGTACATGGTCAGGAGTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGA
 S  R  T  W  S  G  V  C  G  N  N  N  A  C  K  N  Q  C  I  R

CTTGAGAAAGCACGACATGGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATCTGC
 L  E  K  A  R  H  G  S  C  N  Y  R  F  P  A  H  K  C  I  C

TACTTTCCTTGTTAATAGGAGCTC                (SEQ ID NO 32)
 Y  F  P  C  -  -                       (SEQ ID NO 33)
```

pFAJ3345

Fig.26.

```
 NcoI
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
   M  V  N  R  S  V  A  F  S  A  F  V  L  I  L  F  V  L  A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
 I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S  K  T  W  S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
 G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W  E  G  A  A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTTCC
 H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y  F  N  C  S

AACGCGGCCGACGAGGTGGCTACCCCAGAGGACCAGAAGTTGTGCCAAAGGCCAAGTCGT
 N  A  A  D  E  V  A  T  P  E  D  Q  K  L  C  Q  R  P  S  R

ACATGGTCAGGAGTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAG
 T  W  S  G  V  C  G  N  N  N  A  C  K  N  Q  C  I  R  L  E

AAAGCACGACATGGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATCTGCTACTTT
 K  A  R  H  G  S  C  N  Y  R  F  P  A  H  K  C  I  C  Y  F
                   SacI
CCTTGTTAATAGGAGCTC                (SEQ ID NO 34)
 P  C  -  -                        (SEQ ID NO 35)
```

pFAJ3346

Fig.27.

NcoI
```
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
   M  V  N  R  S  V  A  F  S  A  F  V  L  I  L  F  V  L  A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
 I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S  K  T  W  S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
 G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W  E  G  A  A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTTCC
 H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y  F  N  C  S

AACGCGGCCGACGAGGTGGCTACCCCAGAGCAGAAGTTGTGCCAAAGGCCAAGTCGTACA
 N  A  A  D  E  V  A  T  P  E  Q  K  L  C  Q  R  P  S  R  T

TGGTCAGGAGTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAA
 W  S  G  V  C  G  N  N  N  A  C  K  N  Q  C  I  R  L  E  K

GCACGACATGGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATCTGCTACTTTCCT
 A  R  H  G  S  C  N  Y  R  F  P  A  H  K  C  I  C  Y  F  P
         SacI
TGTTAATAGGAGCTC              (SEQ ID NO 36)
 C  -  -                     (SEQ ID NO 37)
```

pFAJ3369

Fig.28.

NcoI
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
M V N R S V A F S A F V L I L F V L A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
I S D I A S V S G E L C E K A S K T W S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
G N C G N T G H C D N Q C K S W E G A A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTGCT
H G A C H V R N G K H M C F C Y F N C **A**

AACGCTGAGGAAGCTGCTGCTGCTATTCCTGAAGCTTCTGAAGAACTTGCTCAAGAAGAA
N A E E A A A A I P E A S E E L A Q E E

GCTCCTGTGTACAGTGAAGATCAGAAGTTGTGCCAAAGGCCAAGTCGTACATGGTCAGGA
A P V Y S E D Q K L C Q R P S R T W S G

GTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAAGCACGACAT
V C G N N N A C K N Q C I R L E K A R H

GGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATCTGCTACTTTCCTTGTTAATAG
G S C N Y R F P A H K C I C Y F P C - -
SacI
GAGCTC

(SEQ ID NO 38)
(SEQ ID NO 39)

Fig.29.

pFAJ3367

NcoI

```
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
   M  V  N  R  S  V  A  F  S  A  F  V  L  I  L  F  V  L  A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
 I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S  K  T  W  S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
 G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W  E  G  A  A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAACTGCGCT
 H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y  F  N  C  A

AACGCTGAGGAAGCTGCTGCTGCTATTCCTGAAGCTTCTGAAGAACTTGCTCAAGAAGAA
 N  A  E  E  A  A  A  A  I  P  E  A  S  E  E  L  A  Q  E  E

GCTCCTGTGTACAGTGAAGATCAGAAGTTGTGCCAAAGGCCAAGTCGTACATGGTCAGGA
 A  P  V  Y  S  E  D  Q  K  L  C  Q  R  P  S  R  T  W  S  G

GTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAAGCACGACAT
 V  C  G  N  N  N  A  C  K  N  Q  C  I  R  L  E  K  A  R  H

GGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATCTGCTACTTCCCTTGTGCGAAT
 G  S  C  N  Y  R  F  P  A  H  K  C  I  C  Y  F  P  C  A  N

GCTGAAGAAGCTGCTGCTGCTATTCCTGAAGCTTCTGAAGAACTTGCTCAAGAAGAAGCA
 A  E  E  A  A  A  A  I  P  E  A  S  E  E  L  A  Q  E  E  A

CCGGTTTACTCTGAAGATGACGGAGTGAAGCTCTGCGACGTGCCATCCGGAACCTGGTCC
 P  V  Y  S  E  D  D  G  V  K  L  C  D  V  P  S  G  T  W  S

GGACACTGCGGTTCCTCCAGCAAGTGCAGCCAACAATGCAAGGACAGGGAGCACTTCGCT
 G  H  C  G  S  S  S  K  C  S  Q  Q  C  K  D  R  E  H  F  A

TACGGAGGAGCTTGCCACTACCAATTCCCATCCGTGAAGTGCTTCTGCAAGAGGCAATGC
 Y  G  G  A  C  H  Y  Q  F  P  S  V  K  C  F  C  K  R  Q  C

GCTAACGCTGAGGAAGCTGCTGCTGCTATTCCTGAAGCTTCTGAAGAACTTGCTCAAGAA
 A  N  A  E  E  A  A  A  A  I  P  E  A  S  E  E  L  A  Q  E

GAAGCTCCTGTGTACAGTGAAGATCAGAACATATGCCCAAGGGTTAATCGAATTGTGACA
 E  A  P  V  Y  S  E  D  Q  N  I  C  P  R  V  N  R  I  V  T

CCCTGTGTGGCCTACGGACTCGGAAGGGCACCAATCGCCCCATGCTGCAGAGCCCTGAAC
 P  C  V  A  Y  G  L  G  R  A  P  I  A  P  C  C  R  A  L  N

GATCTACGGTTTGTGAATACTAGAAACCTACGACGTGCTGCATGCCGCTGCCTCGTAGGG
 D  L  R  F  V  N  T  R  N  L  R  R  A  A  C  R  C  L  V  G

GTAGTGAACCGGAACCCCGGTCTGAGACGAAACCCTAGATTTCAGAACATTCCTCGTGAT
 V  V  N  R  N  P  G  L  R  R  N  P  R  F  Q  N  I  P  R  D

TGTCGCAACACCTTTGTTCGTCCCTTCTGGTGGCGTCCAAGAATTCAATGCGGCAGGATT
 C  R  N  T  F  V  R  P  F  W  W  R  P  R  I  Q  C  G  R  I
     SacI
AACTAATAGAGCTC          (SEQ ID NO 40)
 N  -  -                (SEQ ID NO 41)
```

Fig.30.

pFAJ3106-2

NcoI

CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
M V N R S V A F S A F V L I L F V L A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
I S D I A S V S G E L C E K A S K T W S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
G N C G N T G H C D N Q C K S W E G A A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTAAA
H G A C H V R N G K H M C F C Y F N C **K**

AAAGCCGAAAAGCTTGCTCAAGACAAACTTAAAGCCGAACAACTCATCGGAAAGAGGATC
K A E K L A Q D K L K A E Q L I G K R I

GGAAAGAGGATCGGAAAGAGGCAGAAGTTGTGCCAAAGGCCAAGTCGTACATGGTCAGGA
G K R I G K R Q K L C Q R P S R T W S G

GTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAAGCACGACAT
V C G N N N A C K N Q C I R L E K A R H

GGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATCTGCTACTTTCCTTGTTAATAG
G S C N Y R F P A H K C I C Y F P C - -

SacI

GAGCTC

(SEQ ID NO 42)
(SEQ ID NO 43)

Fig.31.

pFAJ3107-2

```
 NcoI
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
   M  V  N  R  S  V  A  F  S  A  F  V  L  I  L  F  V  L  A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
 I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S  K  T  W  S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
 G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W  E  G  A  A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTAAA
 H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y  F  N  C  K

AAAGCCGAAAAAGCTTGCTCAAGACAAACTTAAAGCCGAACAACTCGCTCAAGACAAACTT
 K  A  E  K  L  A  Q  D  K  L  K  A  E  Q  L  A  Q  D  K  L

AATGCCCAAAAGCTTGACCGTGATGCCAAGAAAGTGGTTCCAAACGTTGAACATCCGATC
 N  A  Q  K  L  D  R  D  A  K  K  V  V  P  N  V  E  H  P  I

GGAAAGAGGATCGGAAAGAGGATCGGAAAGAGGCAGAAGTTGTGCCAAAGGCCAAGTCGT
 G  K  R  I  G  K  R  I  G  K  R  Q  K  L  C  Q  R  P  S  R

ACATGGTCAGGAGTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAG
 T  W  S  G  V  C  G  N  N  N  A  C  K  N  Q  C  I  R  L  E

AAAGCACGACATGGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATCTGCTACTT
 K  A  R  H  G  S  C  N  Y  R  F  P  A  H  K  C  I  C  Y  F
                  SacI
TCCTTGTTAATAGGAGCTC           (SEQ ID NO 44)
 P  C  -  -                   (SEQ ID NO 45)
```

pFAJ3108-2

Fig.32.

NcoI
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
M V N R S V A F S A F V L I L F V L A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
I S D I A S V S G E L C E K A S K T W S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
G N C G N T G H C D N Q C K S W E G A A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTGCC
H G A C H V R N G K H M C F C Y F N C **A**

AGTACTACTGTGGATCACCAAGCTGATGTTGCTGCCACCAAAACTATCGGAAAGAGGATC
S T T V D H Q A D V A A T K T ***I G K R I***

GGAAAGAGGATCGGAAAGAGGCAGAAGTTGTGCCAAAGGCCAAGTCGTACATGGTCAGGA
G K R I G K R Q K L C Q R P S R T W S G

GTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAAGCACGACAT
V C G N N N A C K N Q C I R L E K A R H

GGATCTTGCAACTATCTGTTCCCAGCTCACAAGTGTATCTGCTACTTTCCTTGTTAATAG
G S C N Y R F P A H K C I C Y F P C - -

SacI
GAGCTC (SEQ ID NO 46)
(SEQ ID NO 47)

pFAJ3370

Fig.33.

```
 NcoI
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
   M  V  N  R  S  V  A  F  S  A  F  V  L  I  L  F  V  L  A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
 I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S  K  T  W  S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
 G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W  E  G  A  A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTTCC
 H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y  F  N  C  S

AACGCGGCCGACGAGGTGGCTACCCAGCTGTTGAATTTTGACCTTCTTAAGCTTGCGGGA
 N  A  A  D  E  V  A  T  Q  L  L  N  F  D  L  L  K  L  A  G

GACGTCGAGTCCAACCCTGGGCCCCAGAAGTTGTGCCAAAGGCCAAGTCGTACATGGTCA
 D  V  E  S  N  P  G  P  Q  K  L  C  Q  R  P  S  R  T  W  S

GGAGTCTGTGGAAACAATAACGCATGCAAGAATCAGTGCATTAGACTTGAGAAAGCACGA
 G  V  C  G  N  N  N  A  C  K  N  Q  C  I  R  L  E  K  A  R

CATGGATCTTGCAACTATCGTTTCCCAGCTCACAAGTGTATCTGCTACTTTCCTTGTTAA
 H  G  S  C  N  Y  R  F  P  A  H  K  C  I  C  Y  F  P  C  -
  SacI
TAGGAGCTC              (SEQ ID NO 48)
                       (SEQ ID NO 49)
 -
```

pFAJ3368

Fig.34.

```
 NcoI
CCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTTCTGATCCTTTTCGTGCTCGCC
   M  V  N  R  S  V  A  F  S  A  F  V  L  I  L  F  V  L  A

ATCTCAGATATCGCATCCGTTAGTGGAGAACTATGCGAGAAAGCTAGCAAGACGTGGTCG
 I  S  D  I  A  S  V  S  G  E  L  C  E  K  A  S  K  T  W  S

GGCAACTGTGGCAACACGGGACATTGTGACAACCAATGTAAATCATGGGAGGGTGCGGCT
 G  N  C  G  N  T  G  H  C  D  N  Q  C  K  S  W  E  G  A  A

CACGGAGCGTGTCATGTGCGTAACGGGAAACACATGTGTTTCTGTTACTTCAATTGTTCC
 H  G  A  C  H  V  R  N  G  K  H  M  C  F  C  Y  F  N  C  S

AACGCGGCCGACGAGGTGGCTACCCAGCTGTTGAATTTTGACCTTCTTAAGCTTGCGGGA
 N  A  A  D  E  V  A  T  Q  L  L  N  F  D  L  L  K  L  A  G

GACGTCGAGTCCAACCCTGGGCCCATGGCTAAGTTTGCGTCCATCATCGCACTTCTTTTT
 D  V  E  S  N  P  G  P  M  A  K  F  A  S  I  I  A  L  L  F

GCTGCTCTTGTTCTTTTTGCTGCTTTCGAAGCACCAACAATGGTGGAAGCACAGAAGTTG
 A  A  L  V  L  F  A  A  F  E  A  E  T  M  V  E  A  Q  K  L

TGCCAAAGGCCAAGTCGTACATGGTCAGGAGTCTGTGGAAACAATAACGCATGCAAGAAT
 C  Q  R  P  S  R  T  W  S  G  V  C  G  N  N  N  A  C  K  N

CAGTGCATTAGACTTGAGAAAGCACGACATGGATCTTGCAACTATCGTTTCCCAGCTCAC
 Q  C  I  R  L  E  K  A  R  H  G  S  C  N  Y  R  F  P  A  H
                                          SacI
AAGTGTATCTGCTACTTTCCTTGTTAATAGGAGCTC                 (SEQ ID NO 50)
 K  C  I  C  Y  F  P  C  -  -                        (SEQ ID NO 51)
```

Docket No.
PPD 50348/UST

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

GENETIC METHOD FOR THE EXPRESSION OF POLYPROTEINS IN PLANTS

the specification of which

______ is attached hereto
X was filed on 17th August 1999 as PCT International Application Number GB99/02716 and was amended on ______ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed Yes	Priority Claimed No
9818001.1	United Kingdom	18th August 1998	X	
9826753.7	United Kingdom	4th December 1998	X	

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT International Application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application.

Application Number	Filing Date	Status

2

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:
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1800 Concord Pike
P.O. Box 15458
Wilmington, DE 19850-5458

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature J A Ray Date 27th February 2001

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Inventor's signature Isabelle E.J.A. François Date 12 february 2001

Residence Heverlee, Belgium BEX Belgian

Post Office Address F. A. Janssen's Laboratory of Genetics, Katholieke Universeit Leuven, Kardinaal, Mercierlaan 92, B3001 Heverlee-Leuven, Belgium

4-00 Full name of fourth inventor, if any (given name, family name) Miguel Francesco Coleta DE BOLLE

Inventor's signature Miguel F.C. De Bolle Date 12 february 2001

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Post Office Address F. A. Janssen's Laboratory of Genetics, Katholieke Universeit Leuven, Kardinaal, Mercierlaan 92, B3001 Heverlee-Leuven, Belgium

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Inventor's signature Willem Frans Broekaert Date 8 february 2001

Residence Gent, Belgium BEX Belgian

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Application

Address to:
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Washington, D.C. 20231

Application Number	09/763,076
Filing Date	To Be Determined
First Named Inventor	Broekaert et al.
Group Art Unit	Unassigned
Examiner Name	Unassigned
Attorney Docket Number	SYN-071 (109.846.205)

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☐ Assignee of record of the entire interest.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☑ Attorney or Agent of record.

☐ Registered practitioner named in the application transmittal letter in an application without an executed oath or declaration. See 37 CFR 1.33(a)(1). Registration Number ____________

Typed or Printed Name	Robert McIsaac, Ph.D., Registration No. 46,918
Signature	Robert McIsaac
Date	May 14, 2001

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

[X] *Total of 1 forms are submitted.

SEQUENCE LISTING

<110> ZENECA Limited
Broekaert, Willem F
Francois, Isabelle EJA
Evans, Ian J
De Bolle, Miguel FC
Ray, John A

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Val Leu Ala Ile Ser
            20

ttatattctt ttatggtttt atgtgttctg acaagttgca aatattgagt ag at atc  161
                                                          Asp Ile

gca tcc gtt agt gga gaa cta tgc gag aaa gct agc aag aca tgg tcg   209
Ala Ser Val Ser Gly Glu Leu Cys Glu Lys Ala Ser Lys Thr Trp Ser
     25                  30                  35

gga aac tgt ggc aat acg gga cat tgt gac aac caa tgt aaa tca tgg   257
Gly Asn Cys Gly Asn Thr Gly His Cys Asp Asn Gln Cys Lys Ser Trp
 40                  45                  50                  55

gag ggt gcg gcc cat gga gcg tgt cat gtg cgt aac ggg aaa cac atg   305
Glu Gly Ala Ala His Gly Ala Cys His Val Arg Asn Gly Lys His Met
                 60                  65                  70

tgt ttc tgt tac ttc aat tgt aaa aaa gcc gaa aag ctt gct caa gac   353
Cys Phe Cys Tyr Phe Asn Cys Lys Lys Ala Glu Lys Leu Ala Gln Asp
             75                  80                  85

aaa ctt aaa gcc gaa caa ctc gct caa gac aaa ctt aat gcc caa aag   401
Lys Leu Lys Ala Glu Gln Leu Ala Gln Asp Lys Leu Asn Ala Gln Lys
         90                  95                 100

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<213> Dahlia merckii

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            20                  25                  30

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        35                  40                  45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
    50                  55                  60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Lys Lys
65                  70                  75                  80

Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu Ala Gln
                85                  90                  95

Asp Lys Leu Asn Ala Gln Lys Leu Asp Arg Asp Ala Lys Lys Val Val
            100                 105                 110

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1 5 10 15

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Ile Gly Lys Arg
20

<210> 5
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<213> Dahlia merckii

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20 25 30

Val Val Pro Asn Val Glu His Pro
35 40

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20

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<213> Amaranthus caudatus

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Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val
1 5 10

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Glu Leu Cys Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn
    30                  35                  40

acg gga cat tgt gac aac caa tgt aaa tca tgg gag ggt gcg gcc cat    255
Thr Gly His Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His
45                  50                  55                  60

gga gcg tgt cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc    303
Gly Ala Cys His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe
                65                  70                  75

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Asn Cys Ser Asn Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Val Glu
            80                  85                  90

cca gga cag aag ttg tgc caa agg cca agt ggg aca tgg tca gga gtc    399
Pro Gly Gln Lys Leu Cys Gln Arg Pro Ser Gly Thr Trp Ser Gly Val
        95                  100                 105

tgt gga aac aat aac gca tgc aag aat cag tgc att aga ctt gag aaa    447
Cys Gly Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys
    110                 115                 120

gca cga cat gga tct tgc aac tat gtc ttc cca gct cac aag tgt atc    495
Ala Arg His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile
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                145
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             20                  25                  30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
         35                  40                  45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
     50                  55                  60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser Asn
 65                  70                  75                  80

Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Val Glu Pro Gly Gln Lys
                 85                  90                  95

Leu Cys Gln Arg Pro Ser Gly Thr Trp Ser Gly Val Cys Gly Asn Asn
            100                 105                 110

Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly
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    130                 135                 140

Cys
145
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                 Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val
                   1               5                  10

ctg atc ctt ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga   159
Leu Ile Leu Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly
        15                  20                  25

gaa cta tgc gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac   207
Glu Leu Cys Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn
    30                  35                  40

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Thr Gly His Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His
45                  50                  55                  60

gga gcg tgt cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc   303
Gly Ala Cys His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe
                65                  70                  75
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aat tgt aaa aaa gcc gaa aag ctt gct caa gac aaa ctt aaa gcc gaa 351
Asn Cys Lys Lys Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu
80 85 90

caa ctc atc gga aag agg cag aag ttg tgc caa agg cca agt ggg aca 399
Gln Leu Ile Gly Lys Arg Gln Lys Leu Cys Gln Arg Pro Ser Gly Thr
95 100 105

tgg tca gga gtc tgt gga aac aat aac gca tgc aag aat cag tgc att 447
Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile
110 115 120

aga ctt gag aaa gca cga cat gga tct tgc aac tat gtc ttc cca gct 495
Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr Val Phe Pro Ala
125 130 135 140

cac aag tgt atc tgc tac ttt cct tgt taa taggagctc 534
His Lys Cys Ile Cys Tyr Phe Pro Cys
145

<210> 12
<211> 149
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 12
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Lys Lys
65 70 75 80

Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu Ile Gly
85 90 95

Lys Arg Gln Lys Leu Cys Gln Arg Pro Ser Gly Thr Trp Ser Gly Val
100 105 110

Cys Gly Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys
115 120 125

Ala Arg His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile
130 135 140

Cys Tyr Phe Pro Cys
145

<210> 13
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oligonucleotide

<220>
<221> misc_feature
<222> (6, 9, 12, 15, 21)
<223> n is any residue

<400> 13
tgyganaang cnwsnaarac ntgg 24

<210> 14
<211> 8
<212> PRT
<213> Dahlia merckii

<400> 14
Cys Glu Lys Ala Ser Lys Thr Trp
1 5

<210> 15
<211> 606
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> CDS
<222> (76)..(597)

<400> 15
ctcgagtatt tttacaacaa ttaccaacaa caacaaacaa caaacaacat tacaattact 60

atttacaatt acacc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt 111
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val
1 5 10

ctg atc ctt ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga 159
Leu Ile Leu Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly
15 20 25

gaa cta tgc gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac 207
Glu Leu Cys Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn
30 35 40

```
acg gga cat tgt gac aac caa tgt aaa tca tgg gag ggt gcg gcc cat     255
Thr Gly His Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His
 45                 50                  55                  60

gga gcg tgt cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc     303
Gly Ala Cys His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe
                65                  70                  75

aat tgt aaa aaa gcc gaa aag ctt gct caa gac aaa ctt aaa gcc gaa     351
Asn Cys Lys Lys Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu
            80                  85                  90

caa ctc gct caa gac aaa ctt aat gcc caa aag ctt gac cgt gat gcc     399
Gln Leu Ala Gln Asp Lys Leu Asn Ala Gln Lys Leu Asp Arg Asp Ala
        95                 100                 105

aag aaa gtg gtt cca aac gtt gaa cat ccg atc gga aag agg cag aag     447
Lys Lys Val Val Pro Asn Val Glu His Pro Ile Gly Lys Arg Gln Lys
    110                 115                 120

ttg tgc caa agg cca agt ggg aca tgg tca gga gtc tgt gga aac aat     495
Leu Cys Gln Arg Pro Ser Gly Thr Trp Ser Gly Val Cys Gly Asn Asn
125                 130                 135                 140

aac gca tgc aag aat cag tgc att aga ctt gag aaa gca cga cat gga     543
Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly
                145                 150                 155

tct tgc aac tat gtc ttc cca gct cac aag tgt atc tgc tac ttt cct     591
Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro
            160                 165                 170

tgt taa taggagctc                                                   606
Cys
```

<210> 16

<211> 173

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence: Synthetic
sequence

<400> 16

Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Lys Lys
65 70 75 80

Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu Ala Gln
85 90 95

Asp Lys Leu Asn Ala Gln Lys Leu Asp Arg Asp Ala Lys Lys Val Val
100 105 110

Pro Asn Val Glu His Pro Ile Gly Lys Arg Gln Lys Leu Cys Gln Arg
115 120 125

Pro Ser Gly Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys
130 135 140

Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr
145 150 155 160

Val Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
165 170

<210> 17
<211> 534
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> CDS
<222> (76)..(525)

<400> 17
ctcgagtatt tttacaacaa ttaccaacaa caacaaacaa caaacaacat tacaattact 60

atttacaatt acacc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt 111
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val
1 5 10

ctg atc ctt ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga 159
Leu Ile Leu Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly
15 20 25

gaa cta tgc gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac 207
Glu Leu Cys Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn
30 35 40

acg gga cat tgt gac aac caa tgt aaa tca tgg gag ggt gcg gcc cat 255
Thr Gly His Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His
45 50 55 60

gga gcg tgt cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc 303
Gly Ala Cys His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe
65 70 75

aat tgt gcc agt act act gtg gat cac caa gct gat gtt gct gcc acc 351
Asn Cys Ala Ser Thr Thr Val Asp His Gln Ala Asp Val Ala Ala Thr
80 85 90

aaa act atc gga aag agg cag aag ttg tgc caa agg cca agt ggg aca 399
Lys Thr Ile Gly Lys Arg Gln Lys Leu Cys Gln Arg Pro Ser Gly Thr
95 100 105

tgg tca gga gtc tgt gga aac aat aac gca tgc aag aat cag tgc att 447
Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile
110 115 120

aga ctt gag aaa gca cga cat gga tct tgc aac tat gtc ttc cca gct 495
Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr Val Phe Pro Ala
125 130 135 140

cac aag tgt atc tgc tac ttt cct tgt taa taggagctc 534
His Lys Cys Ile Cys Tyr Phe Pro Cys
145

<210> 18
<211> 149
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 18
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ala Ser
65 70 75 80

Thr Thr Val Asp His Gln Ala Asp Val Ala Ala Thr Lys Thr Ile Gly
85 90 95

Lys Arg Gln Lys Leu Cys Gln Arg Pro Ser Gly Thr Trp Ser Gly Val
100 105 110

Cys Gly Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys
115 120 125

Ala Arg His Gly Ser Cys Asn Tyr Val Phe Pro Ala His Lys Cys Ile
130 135 140

Cys Tyr Phe Pro Cys
145

<210> 19
<211> 316
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> CDS
<222> (76)..(312)

<400> 19

ctcgagtatt tttacaacaa ttaccaacaa caacaaacaa caaacaacat tacaattact 60

atttacaatt acacc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt 111
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val
1 5 10

ctg atc ctt ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga 159
Leu Ile Leu Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly
15 20 25

gaa cta tgc gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac 207
Glu Leu Cys Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn
30 35 40

acg gga cat tgt gac aac caa tgt aaa tca tgg gag ggt gcg gcc cat 255
Thr Gly His Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His
45 50 55 60

gga gcg tgt cat gtg cgt aat ggg aaa cac atg tgt ttc tgt tac ttc 303
Gly Ala Cys His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe
65 70 75

aat tgt tga gctc 316
Asn Cys

<210> 20
<211> 78
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 20
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys
65 70 75

<210> 21
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Linker peptide

<400> 21
Ser Asn Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Val Glu
1 5 10

<210> 22
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Linker peptide

<400> 22

Ser Asn Ala Ala Asp Glu Val Ala Thr Pro Glu Asp
  1   5   10

<210> 23

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Linker
      peptide

<400> 23

Ser Asn Ala Ala Asp Glu Val Ala Thr Pro Glu
  1   5   10

<210> 24

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Linker
      peptide

<400> 24

Ala Asn Ala Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu
  1   5   10   15

Leu Ala Gln Glu Glu Ala Pro Val Tyr Ser Glu Asp
             20   25

<210> 25
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Linker propeptide

<400> 25

```
Lys Lys Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu
  1               5                  10                  15

Ile Gly Lys Arg Ile Gly Lys Arg Ile Gly Lys Arg
             20                  25
```

<210> 26
<211> 52
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Linker propeptide

<400> 26

```
Lys Lys Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu
  1               5                  10                  15

Ala Gln Asp Lys Leu Asn Ala Gln Lys Leu Asp Arg Asp Ala Lys Lys
             20                  25                  30

Val Val Pro Asn Val Glu His Pro Ile Gly Lys Arg Ile Gly Lys Arg
         35                  40                  45

Ile Gly Lys Arg
     50
```

<210> 27

<211> 28

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Linker
propeptide

<400> 27

```
Ala Ser Thr Thr Val Asp His Gln Ala Asp Val Ala Ala Thr Lys Thr
  1               5                  10                  15

Ile Gly Lys Arg Ile Gly Lys Arg Ile Gly Lys Arg
             20                  25
```

<210> 28

<211> 29

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Linker
propeptide

<400> 28

```
Ser Asn Ala Ala Asp Glu Val Ala Thr Gln Leu Leu Asn Phe Asp Leu
  1               5                  10                  15

Leu Lys Leu Ala Gly Asp Val Glu Ser Asn Pro Gly Pro
             20                  25
```

<210> 29

<211> 15

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Linker peptide

<400> 29

Asn Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Val Glu Pro Gly
1 5 10 15

<210> 30

<211> 446

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
sequence

<220>

<221> CDS

<222> (3)..(437)

<400> 30

cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt 47
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
1 5 10 15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc 95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
20 25 30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat 143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
35 40 45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt 191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
50 55 60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt aac 239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Asn
65 70 75

gcg gcc gac gag gtg gct acc cca gag gac gtg gaa cct ggt cag aag 287
Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Val Glu Pro Gly Gln Lys
80 85 90 95

ttg tgc caa agg cca agt cgt aca tgg tca gga gtc tgt gga aac aat 335
Leu Cys Gln Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn
100 105 110

aac gca tgc aag aat cag tgc att aga ctt gag aaa gca cga cat gga 383
Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly
115 120 125

tct tgc aac tat cgt ttc cca gct cac aag tgt atc tgc tac ttt cct 431
Ser Cys Asn Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro
130 135 140

tgt taa taggagctc 446
Cys

<210> 31
<211> 144
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 31
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Asn Ala
65 70 75 80

Ala Asp Glu Val Ala Thr Pro Glu Asp Val Glu Pro Gly Gln Lys Leu
85 90 95

Cys Gln Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn
100 105 110

Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser
115 120 125

Cys Asn Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
130 135 140

<210> 32

<211> 443

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic sequence

<220>

<221> CDS

<222> (3)..(434)

<400> 32

```
cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt      47
   Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
    1               5                  10                  15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc     95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
                20                  25                  30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat    143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
            35                  40                  45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt    191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
        50                  55                  60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt tcc    239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser
    65                  70                  75

aac gcg gcc gac gag gtg gct acc cca gag gac gtg gaa cag aag ttg    287
Asn Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Val Glu Gln Lys Leu
80                  85                  90                  95

tgc caa agg cca agt cgt aca tgg tca gga gtc tgt gga aac aat aac    335
Cys Gln Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn
                100                 105                 110

gca tgc aag aat cag tgc att aga ctt gag aaa gca cga cat gga tct    383
Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser
            115                 120                 125

tgc aac tat cgt ttc cca gct cac aag tgt atc tgc tac ttt cct tgt    431
Cys Asn Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
        130                 135                 140

taa taggagctc                                                      443
```

<210> 33

<211> 143

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence: Synthetic
sequence

<400> 33

```
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
 1               5                  10                  15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
            20                  25                  30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
        35                  40                  45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
    50                  55                  60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser Asn
65                  70                  75                  80

Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Val Glu Gln Lys Leu Cys
                85                  90                  95

Gln Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala
            100                 105                 110

Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys
        115                 120                 125

Asn Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
    130                 135                 140
```

<210> 34

<211> 437

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic sequence

<220>

<221> CDS

<222> (3)..(428)

<400> 34

```
cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt     47
   Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
     1               5                  10                  15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc     95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
                20                  25                  30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat    143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
            35                  40                  45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt    191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
        50                  55                  60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt tcc    239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser
    65                  70                  75

aac gcg gcc gac gag gtg gct acc cca gag gac cag aag ttg tgc caa    287
Asn Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Gln Lys Leu Cys Gln
 80                  85                  90                  95
```

agg cca agt cgt aca tgg tca gga gtc tgt gga aac aat aac gca tgc 335
Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys
100 105 110

aag aat cag tgc att aga ctt gag aaa gca cga cat gga tct tgc aac 383
Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn
115 120 125

tat cgt ttc cca gct cac aag tgt atc tgc tac ttt cct tgt taa 428
Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
130 135 140

taggagctc 437

<210> 35
<211> 141
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 35
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser Asn
65 70 75 80

30

Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Gln Lys Leu Cys Gln Arg
85 90 95

Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys
100 105 110

Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr
115 120 125

Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
130 135 140

<210> 36
<211> 434
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> CDS
<222> (3)..(425)

<400> 36
cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt 47
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
1 5 10 15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc 95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
20 25 30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat 143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
35 40 45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt 191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
50 55 60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt tcc 239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser
65 70 75

aac gcg gcc gac gag gtg gct acc cca gag cag aag ttg tgc caa agg 287
Asn Ala Ala Asp Glu Val Ala Thr Pro Glu Gln Lys Leu Cys Gln Arg
80 85 90 95

cca agt cgt aca tgg tca gga gtc tgt gga aac aat aac gca tgc aag 335
Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys
100 105 110

aat cag tgc att aga ctt gag aaa gca cga cat gga tct tgc aac tat 383
Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr
115 120 125

cgt ttc cca gct cac aag tgt atc tgc tac ttt cct tgt taa taggagctc 434
Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
130 135 140

<210> 37
<211> 140
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 37
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser Asn
65 70 75 80

Ala Ala Asp Glu Val Ala Thr Pro Glu Gln Lys Leu Cys Gln Arg Pro
85 90 95

Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys Asn
100 105 110

Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr Arg
115 120 125

Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
130 135 140

<210> 38

<211> 485

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic sequence

<220>

<221> CDS

<222> (3)..(476)

<400> 38

cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt 47
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
1 5 10 15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc 95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
20 25 30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat 143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
35 40 45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt 191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
50 55 60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt gct 239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ala
65 70 75

aac gct gag gaa gct gct gct gct att cct gaa gct tct gaa gaa ctt 287
Asn Ala Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu Leu
80 85 90 95

gct caa gaa gaa gct cct gtg tac agt gaa gat cag aag ttg tgc caa 335
Ala Gln Glu Glu Ala Pro Val Tyr Ser Glu Asp Gln Lys Leu Cys Gln
100 105 110

agg cca agt cgt aca tgg tca gga gtc tgt gga aac aat aac gca tgc 383
Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys
115 120 125

aag aat cag tgc att aga ctt gag aaa gca cga cat gga tct tgc aac 431
Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn
130 135 140

tat cgt ttc cca gct cac aag tgt atc tgc tac ttt cct tgt taa 476
Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
145 150 155

taggagctc 485

<210> 39

<211> 157

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence: Synthetic
sequence

<400> 39

Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ala Asn
65 70 75 80

Ala Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu Leu Ala
85 90 95

Gln Glu Glu Ala Pro Val Tyr Ser Glu Asp Gln Lys Leu Cys Gln Arg
100 105 110

Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys
115 120 125

Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr
130 135 140

Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
145 150 155

<210> 40
<211> 1093
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> CDS
<222> (3)..(1085)

<400> 40
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cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt      47
   Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
    1               5                  10                  15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc      95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
                20                  25                  30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat     143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
            35                  40                  45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt     191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
        50                  55                  60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aac tgc gct     239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ala
    65                  70                  75

aac gct gag gaa gct gct gct gct att cct gaa gct tct gaa gaa ctt     287
Asn Ala Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu Leu
80                  85                  90                  95
```

```
gct caa gaa gaa gct cct gtg tac agt gaa gat cag aag ttg tgc caa     335
Ala Gln Glu Glu Ala Pro Val Tyr Ser Glu Asp Gln Lys Leu Cys Gln
                100                 105                 110

agg cca agt cgt aca tgg tca gga gtc tgt gga aac aat aac gca tgc     383
Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys
            115                 120                 125

aag aat cag tgc att aga ctt gag aaa gca cga cat gga tct tgc aac     431
Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn
        130                 135                 140

tat cgt ttc cca gct cac aag tgt atc tgc tac ttc cct tgt gcg aat     479
Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys Ala Asn
    145                 150                 155

gct gaa gaa gct gct gct gct att cct gaa gct tct gaa gaa ctt gct     527
Ala Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu Leu Ala
160                 165                 170                 175

caa gaa gaa gca ccg gtt tac tct gaa gat gac gga gtg aag ctc tgc     575
Gln Glu Glu Ala Pro Val Tyr Ser Glu Asp Asp Gly Val Lys Leu Cys
                180                 185                 190

gac gtg cca tcc gga acc tgg tcc gga cac tgc ggt tcc tcc agc aag     623
Asp Val Pro Ser Gly Thr Trp Ser Gly His Cys Gly Ser Ser Ser Lys
            195                 200                 205

tgc agc caa caa tgc aag gac agg gag cac ttc gct tac gga gga gct     671
Cys Ser Gln Gln Cys Lys Asp Arg Glu His Phe Ala Tyr Gly Gly Ala
        210                 215                 220

tgc cac tac caa ttc cca tcc gtg aag tgc ttc tgc aag agg caa tgc     719
Cys His Tyr Gln Phe Pro Ser Val Lys Cys Phe Cys Lys Arg Gln Cys
    225                 230                 235
```

gct aac gct gag gaa gct gct gct gct att cct gaa gct tct gaa gaa 767
Ala Asn Ala Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu
240 245 250 255

ctt gct caa gaa gaa gct cct gtg tac agt gaa gat cag aac ata tgc 815
Leu Ala Gln Glu Glu Ala Pro Val Tyr Ser Glu Asp Gln Asn Ile Cys
260 265 270

cca agg gtt aat cga att gtg aca ccc tgt gtg gcc tac gga ctc gga 863
Pro Arg Val Asn Arg Ile Val Thr Pro Cys Val Ala Tyr Gly Leu Gly
275 280 285

agg gca cca atc gcc cca tgc tgc aga gcc ctg aac gat cta cgg ttt 911
Arg Ala Pro Ile Ala Pro Cys Cys Arg Ala Leu Asn Asp Leu Arg Phe
290 295 300

gtg aat act aga aac cta cga cgt gct gca tgc cgc tgc ctc gta ggg 959
Val Asn Thr Arg Asn Leu Arg Arg Ala Ala Cys Arg Cys Leu Val Gly
305 310 315

gta gtg aac cgg aac ccc ggt ctg aga cga aac cct aga ttt cag aac 1007
Val Val Asn Arg Asn Pro Gly Leu Arg Arg Asn Pro Arg Phe Gln Asn
320 325 330 335

att cct cgt gat tgt cgc aac acc ttt gtt cgt ccc ttc tgg tgg cgt 1055
Ile Pro Arg Asp Cys Arg Asn Thr Phe Val Arg Pro Phe Trp Trp Arg
340 345 350

cca aga att caa tgc ggc agg att aac taa tagagctc 1093
Pro Arg Ile Gln Cys Gly Arg Ile Asn
355 360

<210> 41
<211> 360
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 41

Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1   5   10   15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
            20   25   30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
        35   40   45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
    50   55   60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ala Asn
65   70   75   80

Ala Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu Leu Ala
                85   90   95

Gln Glu Glu Ala Pro Val Tyr Ser Glu Asp Gln Lys Leu Cys Gln Arg
            100   105   110

Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys
        115   120   125

Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr
    130   135   140

Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys Ala Asn Ala
145   150   155   160

Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu Leu Ala Gln
                165   170   175

Glu Glu Ala Pro Val Tyr Ser Glu Asp Asp Gly Val Lys Leu Cys Asp
            180   185   190

Val Pro Ser Gly Thr Trp Ser Gly His Cys Gly Ser Ser Ser Lys Cys
195 200 205

Ser Gln Gln Cys Lys Asp Arg Glu His Phe Ala Tyr Gly Gly Ala Cys
210 215 220

His Tyr Gln Phe Pro Ser Val Lys Cys Phe Cys Lys Arg Gln Cys Ala
225 230 235 240

Asn Ala Glu Glu Ala Ala Ala Ala Ile Pro Glu Ala Ser Glu Glu Leu
245 250 255

Ala Gln Glu Glu Ala Pro Val Tyr Ser Glu Asp Gln Asn Ile Cys Pro
260 265 270

Arg Val Asn Arg Ile Val Thr Pro Cys Val Ala Tyr Gly Leu Gly Arg
275 280 285

Ala Pro Ile Ala Pro Cys Cys Arg Ala Leu Asn Asp Leu Arg Phe Val
290 295 300

Asn Thr Arg Asn Leu Arg Arg Ala Ala Cys Arg Cys Leu Val Gly Val
305 310 315 320

Val Asn Arg Asn Pro Gly Leu Arg Arg Asn Pro Arg Phe Gln Asn Ile
325 330 335

Pro Arg Asp Cys Arg Asn Thr Phe Val Arg Pro Phe Trp Trp Arg Pro
340 345 350

Arg Ile Gln Cys Gly Arg Ile Asn
355 360

<210> 42

<211> 485

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
sequence

<220>

<221> CDS

<222> (3)..(476)

<400> 42

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cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt      47
   Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
     1               5                  10                  15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc      95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
                20                  25                  30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat     143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
            35                  40                  45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt     191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
        50                  55                  60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt aaa     239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Lys
    65                  70                  75

aaa gcc gaa aag ctt gct caa gac aaa ctt aaa gcc gaa caa ctc atc     287
Lys Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu Ile
80                  85                  90                  95
```

gga aag agg atc gga aag agg atc gga aag agg cag aag ttg tgc caa 335
Gly Lys Arg Ile Gly Lys Arg Ile Gly Lys Arg Gln Lys Leu Cys Gln
100 105 110

agg cca agt cgt aca tgg tca gga gtc tgt gga aac aat aac gca tgc 383
Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys
115 120 125

aag aat cag tgc att aga ctt gag aaa gca cga cat gga tct tgc aac 431
Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn
130 135 140

tat cgt ttc cca gct cac aag tgt atc tgc tac ttt cct tgt taa 476
Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
145 150 155

taggagctc 485

<210> 43
<211> 157
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 43
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

```
Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Lys Lys
 65                  70                  75                  80

Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu Ile Gly
                 85                  90                  95

Lys Arg Ile Gly Lys Arg Ile Gly Lys Arg Gln Lys Leu Cys Gln Arg
            100                 105                 110

Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys
        115                 120                 125

Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr
    130                 135                 140

Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
145                 150                 155




<210> 44
<211> 557
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      sequence

<220>
<221> CDS
<222> (3)..(548)

<400> 44
cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt     47
   Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
     1               5                  10                  15
```

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc 95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
20 25 30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat 143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
35 40 45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt 191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
50 55 60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt aaa 239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Lys
65 70 75

aaa gcc gaa aag ctt gct caa gac aaa ctt aaa gcc gaa caa ctc gct 287
Lys Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu Ala
80 85 90 95

caa gac aaa ctt aat gcc caa aag ctt gac cgt gat gcc aag aaa gtg 335
Gln Asp Lys Leu Asn Ala Gln Lys Leu Asp Arg Asp Ala Lys Lys Val
100 105 110

gtt cca aac gtt gaa cat ccg atc gga aag agg atc gga aag agg atc 383
Val Pro Asn Val Glu His Pro Ile Gly Lys Arg Ile Gly Lys Arg Ile
115 120 125

gga aag agg cag aag ttg tgc caa agg cca agt cgt aca tgg tca gga 431
Gly Lys Arg Gln Lys Leu Cys Gln Arg Pro Ser Arg Thr Trp Ser Gly
130 135 140

gtc tgt gga aac aat aac gca tgc aag aat cag tgc att aga ctt gag 479
Val Cys Gly Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu
145 150 155

aaa gca cga cat gga tct tgc aac tat cgt ttc cca gct cac aag tgt 527
Lys Ala Arg His Gly Ser Cys Asn Tyr Arg Phe Pro Ala His Lys Cys
160 165 170 175

atc tgc tac ttt cct tgt taa taggagctc 557
Ile Cys Tyr Phe Pro Cys
180

<210> 45
<211> 181
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 45
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Lys Lys
65 70 75 80

Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala Glu Gln Leu Ala Gln
85 90 95

Asp Lys Leu Asn Ala Gln Lys Leu Asp Arg Asp Ala Lys Lys Val Val
100 105 110

Pro Asn Val Glu His Pro Ile Gly Lys Arg Ile Gly Lys Arg Ile Gly
115 120 125

Lys Arg Gln Lys Leu Cys Gln Arg Pro Ser Arg Thr Trp Ser Gly Val
130 135 140

Cys Gly Asn Asn Asn Ala Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys
145 150 155 160

Ala Arg His Gly Ser Cys Asn Tyr Arg Phe Pro Ala His Lys Cys Ile
165 170 175

Cys Tyr Phe Pro Cys
180

<210> 46
<211> 485
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> CDS
<222> (3)..(476)

<400> 46
cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt 47
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
1 5 10 15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc 95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
20 25 30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat 143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
35 40 45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt 191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
50 55 60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt gcc 239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ala
65 70 75

agt act act gtg gat cac caa gct gat gtt gct gcc acc aaa act atc 287
Ser Thr Thr Val Asp His Gln Ala Asp Val Ala Ala Thr Lys Thr Ile
80 85 90 95

gga aag agg atc gga aag agg atc gga aag agg cag aag ttg tgc caa 335
Gly Lys Arg Ile Gly Lys Arg Ile Gly Lys Arg Gln Lys Leu Cys Gln
100 105 110

agg cca agt cgt aca tgg tca gga gtc tgt gga aac aat aac gca tgc 383
Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys
115 120 125

aag aat cag tgc att aga ctt gag aaa gca cga cat gga tct tgc aac 431
Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn
130 135 140

tat ctg ttc cca gct cac aag tgt atc tgc tac ttt cct tgt taa 476
Tyr Leu Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
145 150 155

taggagctc 485

<210> 47

<211> 157

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence: Synthetic
sequence

<400> 47

Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1   5   10   15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
            20   25   30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
        35   40   45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
    50   55   60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ala Ser
65   70   75   80

Thr Thr Val Asp His Gln Ala Asp Val Ala Ala Thr Lys Thr Ile Gly
                85   90   95

Lys Arg Ile Gly Lys Arg Ile Gly Lys Arg Gln Lys Leu Cys Gln Arg
            100   105   110

Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys
        115   120   125

Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr
    130   135   140

Leu Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
145   150   155

<210> 48

<211> 488

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
sequence

<220>

<221> CDS

<222> (3)..(479)

<400> 48

cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt 47
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
1 5 10 15

ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc 95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
20 25 30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat 143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
35 40 45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt 191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
50 55 60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt tcc 239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser
65 70 75

aac gcg gcc gac gag gtg gct acc cag ctg ttg aat ttt gac ctt ctt 287
Asn Ala Ala Asp Glu Val Ala Thr Gln Leu Leu Asn Phe Asp Leu Leu
80 85 90 95

aag ctt gcg gga gac gtc gag tcc aac cct ggg ccc cag aag ttg tgc 335
Lys Leu Ala Gly Asp Val Glu Ser Asn Pro Gly Pro Gln Lys Leu Cys
100 105 110

caa agg cca agt cgt aca tgg tca gga gtc tgt gga aac aat aac gca 383
Gln Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala
115 120 125

tgc aag aat cag tgc att aga ctt gag aaa gca cga cat gga tct tgc 431
Cys Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys
130 135 140

aac tat cgt ttc cca gct cac aag tgt atc tgc tac ttt cct tgt taa 479
Asn Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
145 150 155

taggagctc 488

<210> 49
<211> 158
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 49
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser Asn
65 70 75 80

Ala Ala Asp Glu Val Ala Thr Gln Leu Leu Asn Phe Asp Leu Leu Lys
85 90 95

Leu Ala Gly Asp Val Glu Ser Asn Pro Gly Pro Gln Lys Leu Cys Gln
100 105 110

Arg Pro Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys
115 120 125

Lys Asn Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn
130 135 140

Tyr Arg Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
145 150 155

<210> 50
<211> 575
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<220>
<221> CDS
<222> (3)..(566)

<400> 50
cc atg gtg aat cgg tcg gtt gcg ttc tcc gcg ttc gtt ctg atc ctt 47
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu
1 5 10 15

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ttc gtg ctc gcc atc tca gat atc gca tcc gtt agt gga gaa cta tgc    95
Phe Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys
                20                  25                  30

gag aaa gct agc aag acg tgg tcg ggc aac tgt ggc aac acg gga cat    143
Glu Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His
            35                  40                  45

tgt gac aac caa tgt aaa tca tgg gag ggt gcg gct cac gga gcg tgt    191
Cys Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys
        50                  55                  60

cat gtg cgt aac ggg aaa cac atg tgt ttc tgt tac ttc aat tgt tcc    239
His Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser
    65                  70                  75

aac gcg gcc gac gag gtg gct acc cag ctg ttg aat ttt gac ctt ctt    287
Asn Ala Ala Asp Glu Val Ala Thr Gln Leu Leu Asn Phe Asp Leu Leu
80                  85                  90                  95

aag ctt gcg gga gac gtc gag tcc aac cct ggg ccc atg gct aag ttt    335
Lys Leu Ala Gly Asp Val Glu Ser Asn Pro Gly Pro Met Ala Lys Phe
                100                 105                 110

gcg tcc atc atc gca ctt ctt ttt gct gct ctt gtt ctt ttt gct gct    383
Ala Ser Ile Ile Ala Leu Leu Phe Ala Ala Leu Val Leu Phe Ala Ala
            115                 120                 125

ttc gaa gca cca aca atg gtg gaa gca cag aag ttg tgc caa agg cca    431
Phe Glu Ala Pro Thr Met Val Glu Ala Gln Lys Leu Cys Gln Arg Pro
        130                 135                 140

agt cgt aca tgg tca gga gtc tgt gga aac aat aac gca tgc aag aat    479
Ser Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys Asn
    145                 150                 155
```

cag tgc att aga ctt gag aaa gca cga cat gga tct tgc aac tat cgt 527
Gln Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr Arg
160 165 170 175

ttc cca gct cac aag tgt atc tgc tac ttt cct tgt taa taggagctc 575
Phe Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
180 185

<210> 51
<211> 187
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 51
Met Val Asn Arg Ser Val Ala Phe Ser Ala Phe Val Leu Ile Leu Phe
1 5 10 15

Val Leu Ala Ile Ser Asp Ile Ala Ser Val Ser Gly Glu Leu Cys Glu
20 25 30

Lys Ala Ser Lys Thr Trp Ser Gly Asn Cys Gly Asn Thr Gly His Cys
35 40 45

Asp Asn Gln Cys Lys Ser Trp Glu Gly Ala Ala His Gly Ala Cys His
50 55 60

Val Arg Asn Gly Lys His Met Cys Phe Cys Tyr Phe Asn Cys Ser Asn
65 70 75 80

Ala Ala Asp Glu Val Ala Thr Gln Leu Leu Asn Phe Asp Leu Leu Lys
85 90 95

Leu Ala Gly Asp Val Glu Ser Asn Pro Gly Pro Met Ala Lys Phe Ala
100 105 110

Ser Ile Ile Ala Leu Leu Phe Ala Ala Leu Val Leu Phe Ala Ala Phe
115 120 125

Glu Ala Pro Thr Met Val Glu Ala Gln Lys Leu Cys Gln Arg Pro Ser
130 135 140

Arg Thr Trp Ser Gly Val Cys Gly Asn Asn Asn Ala Cys Lys Asn Gln
145 150 155 160

Cys Ile Arg Leu Glu Lys Ala Arg His Gly Ser Cys Asn Tyr Arg Phe
165 170 175

Pro Ala His Lys Cys Ile Cys Tyr Phe Pro Cys
180 185

<210> 52
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<221> misc_feature
<222> (9, 12, 15)
<223> n is any residue

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 52
carttraant ancanaaarc acat 24

<210> 53
<211> 8
<212> PRT
<213> Dahlia merckii

<400> 53
Met Cys Phe Cys Tyr Phe Asn Cys
1 5

<210> 54
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 54
aaacacatgt gtttcccatt 20

<210> 55
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 55
agcgtgtcat gtgcgtaat 19

<210> 56
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 56
taaagaaacc gaccctttca cgg 23

<210> 57
<211> 107
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 57
atgcatccat ggtgaatcgg tcggttgcgt tctccgcgtt cgttctgatc cttttcgtgc 60
tcgccatctc agatatcgca tccgttagtg gagaactatg cgagaaa 107

<210> 58
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 58
aaaccgaccg agctcacgga tgttcaacgt ttggaac 37

<210> 59

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 59

agcaagcttt tcgggagctc aacaattgaa gtaa 34

<210> 60

<211> 89

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 60

gcctttggca caacttctgt cctggctcca cgtcctctgg ggtagccacc tcgtcagcag 60

cgttggaaca attgaagtaa cagaaacac 89

<210> 61

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 61

ttagagctcc tattaacaag gaaagtagc 29

<210> 62

<211> 55

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 62

gcctttggca caacttctgc ctctttccga tgagttgttc ggctttaagt ttgtc 55

<210> 63

<211> 53

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 63

gcctttggca caacttctgc ctctttccga tcggatgttc aacgtttgga acc 53

<210> 64

<211> 101

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 64

gcctttggca caacttctgc ctctttccga tagttttggt ggcagcaaca tcagcttggt 60
gatccacagt agtactggca caattgaagt aacagaaaca c 101

<210> 65
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 65
Lys Asp Glu Leu
1

<210> 66
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<221> misc_feature
<222> (9, 12, 21)
<223> n is any residue

<220>
<223> Description of Artificial Sequence:
Oligonucleotide

<400> 66
atggcsaanm rntcrgttgc ntt 23

<210> 67
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
sequence

<400> 67
Ile Gly Lys Arg
1

<210> 68
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 68
aggaagttca tttcatttgg 20

<210> 69
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Determined
N-terminal sequence

<400> 69
Glu Leu Cys Glu Lys Ala Ser
1 5

<210> 70

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Determined
N-terminal sequence

<400> 70

Asp Val Glu Pro Gly Gln Lys
1 5

<210> 71

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Determined
N-terminal sequence

<400> 71

Leu Ile Gly Lys Arg Gln Lys
1 5

<210> 72

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Predicted
C-terminal sequence

<400> 72

Cys Tyr Phe Asn Cys Ser
1 5

<210> 73

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Predicted
C-terminal sequence

<400> 73

Ile Cys Tyr Phe Pro Cys
1 5

<210> 74

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Predicted
C-terminal sequence

<400> 74

Cys Tyr Phe Asn Pro Ser
1 5

<210> 75

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Predicted
C-terminal sequence

<400> 75

Cys Tyr Phe Asn Cys Lys
1 5

<210> 76

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Predicted
C-terminal sequence

<400> 76

Cys Tyr Phe Asn Cys Ala
1 5

<210> 77

<211> 12

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
sequence

<400> 77

Ile Gly Lys Arg Ile Gly Lys Arg Ile Gly Lys Arg
1 5 10

<210> 78

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic sequence

<400> 78

Val Ser Gly Glu Leu Cys
1 5

<210> 79

<211> 22

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic sequence

<400> 79

Phe Asn Cys Ser Asn Ala Ala Asp Glu Val Ala Thr Pro Glu Asp Val
1 5 10 15

Glu Pro Gly Gln Lys Leu
20

<210> 80

<211> 26

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
sequence

<400> 80

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Phe Asn Cys Lys Lys Ala Glu Lys Leu Ala Gln Asp Lys Leu Lys Ala
  1               5                  10                  15

Glu Gln Leu Ile Gly Lys Arg Gln Lys Leu
             20                  25
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<210> 81

<211> 26

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
sequence

<400> 81

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Phe Asn Cys Ala Ser Thr Thr Val Asp His Gln Ala Asp Val Ala Ala
  1               5                  10                  15

Thr Lys Thr Ile Gly Lys Arg Gln Lys Leu
             20                  25
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